Apyrase (Nucleoside Triphosphate-Diphosphohydrolase) and Extracellular Nucleotides Regulate Cotton Fiber Elongation in Cultured Ovules^{1[W][OA]}

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Ectoapyrase enzymes remove the terminal phosphate from extracellular nucleoside tri- and diphosphates. In Arabidopsis (*Arabidopsis thaliana*), two ectoapyrases, AtAPY1 and AtAPY2, have been implicated as key modulators of growth. In fibers of cotton (*Gossypium hirsutum*), transcript levels for GhAPY1 and GhAPY2, two closely related ectoapyrases that have high sequence similarity to AtAPY1 and AtAPY2, are up-regulated when fibers enter their rapid growth phase. In an ovule culture system, fibers release ATP as they grow, and when their ectoapyrase activity is blocked by the addition of polyclonal antiapyrase antibodies or by two different small molecule inhibitors, the medium ATP level rises and fiber growth is suppressed. High concentrations of the poorly hydrolyzable nucleotides ATP γ S and ADP β S applied to the medium inhibit fiber growth, and low concentrations of them stimulate growth, but treatment with adenosine 5'-*O*-thiomonophosphate causes no change in the growth rate. Both the inhibition and stimulation of growth by applied nucleotides can be blocked by an antagonist that blocks purinoceptors in animal cells, and by adenosine. Treatment of cotton ovule cultures with ATP γ S induces increased levels of ethylene, and two ethylene antagonists, aminovinylglycine and silver nitrate, block both the growth stimulatory and growth inhibitory effects of applied nucleotides. In addition, the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid, lowers the concentration of nucleotide needed to promote fiber growth. These data indicate that ectoapyrases and extracellular nucleotides play a significant role in regulating cotton fiber growth and that ethylene is a likely downstream component of the signaling pathway.

Cotton (*Gossypium hirsutum*) fibers are some of the longest single cells in the plant kingdom and are considered a model system for studying cell growth and primary cell wall deposition (Kim and Triplett, 2001). Upland cotton is a widely studied species that has been used for numerous genetic and physiological studies on fiber growth (Wilkins and Arpat, 2005; Shi et al., 2006; Chen et al., 2007; Gao et al., 2007; Lee et al., 2007; Taliercio and Boykin, 2007). Cotton fibers differentiate from the epidermis of the ovule and then undergo cell expansion during the elongation phase of growth from 3 to 16 DPA (Basra and Malik, 1984; Tiwari and Wilkins, 1995) and grow via diffuse

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growth, which does not share the common ultrastructural characteristics found in tip-growing cells (Tiwari and Wilkins, 1995).

Fiber growth can be conveniently studied in cultured ovules, which allows for testing the effects of various growth regulators on the initiation and elongation processes (Kim and Triplett, 2001). For example, Shi et al. (2006) documented the role of ethylene in regulating the growth of cotton fibers in ovule culture, and Qin et al. (2007) showed that very-long-chain fatty acids promoted cotton fiber cell elongation in ovule culture by activating ethylene synthesis.

Ectoapyrases (ecto-nucleoside triphosphate-diphosphohydrolases) are well characterized in animal cells, where they play a key role in reducing the concentration of extracellular nucleotides (e.g. eATP and eADP), which function as signaling agents to activate purinoceptors and induce diverse physiological responses, ranging from neurotransmission to programmed cell death (Burnstock, 2008; Zebisch and Sträter, 2008). Plant apyrases contain four apyrase conserved regions that show sequence similarity with apyrase conserved regions from animal apyrases and serve as a signature motif for this family of proteins (Smith and Kirley, 1999; Kawahara et al., 2003; Kozakiewicz et al., 2008). In Arabidopsis (Arabidopsis thaliana), two ectoapyrase enzymes, AtAPY1 and AtAPY2, play a critical role in controlling the growth of all Arabidopsis tissues tested (Wu et al., 2007). More recently Riewe et al. (2008)

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showed that apyrases mediate potato (Solanum tuberosum) tuber growth. In Arabidopsis these enzymes are most strongly expressed in rapidly growing tissues, such as etiolated hypocotyls, pollen tubes, and the elongation zone of roots (Wu et al., 2007). This raises the question of whether the nucleotide substrates of ectoapyrases are present in the extracellular matrix (ECM) of these tissues. Kim et al. (2006) used a recombinant hybrid reporter protein (luciferase with a cellulose-binding domain attached) to visualize ATP in the ECM of plant cells and observed that the highest levels of eATP are found in the expanding walls of actively growing plant cells, such as root hairs. These results suggest that plant cells, like animal cells, release ATP into the ECM via fusion of secretory vesicles to the plasma membrane during their growth. Wound (Song et al., 2006) and mechanical stimuli (Jeter et al., 2004; Weerasinghe et al., 2009) also result in the release of cellular ATP into the ECM.

A recent review summarizes the evidence that eATP and the ectoapyrases that lower its concentration can regulate growth and other responses in plants (Roux and Steinebrunner, 2007). These findings led us to hypothesize that ectoapyrase activity could affect the growth of cotton fibers. In this study we found that the expression of two cotton apyrases that most resemble AtAPY1 and AtAPY2 is highest during the rapid growth phase of the fibers, and that inhibition of apyrase activity by the addition of chemical apyrase inhibitors or anti-APY1/APY2 antibodies to cotton ovule cultures can inhibit cotton fiber growth. Moreover, applied nucleotides can promote or inhibit cotton fiber growth in a dose-dependent manner, and these effects are blocked by the same chemical antagonists that block the effects of extracellular nucleotides in animals. Our results indicate that extracellular nucleotides and ectoapyrases play an important regulatory role during cotton fiber growth.

RESULTS

Characterization of Two Cotton Apyrases (GhAPY1 and GhAPY2)

The deduced amino acid sequences of GhAPY1 and GhAPY2 are 471 and 469 amino acids in length, respectively. Both cotton apyrases possess all four of the characteristic apyrase conserved regions. An alignment of the deduced amino acid sequence of the coding regions of GhAPY1, GhAPY2, AtAPY1, and AtAPY2 shows that GhAPY1 and GhAPY2 are very closely related with 86% identity (Supplemental Fig. S1). The two cotton apyrases also show significant sequence similarity, with the Arabidopsis ectoapyrases, sharing 67% to 68% identity with AtAPY1 and AtAPY2. Within the apyrase conserved regions, however, GhAPY1 and AtAPY1 share 97.5% identity, while GhAPY1 and AtAPY2 share 92.5% identity. A query of the predicted full cotton apyrase amino acid sequences

using the SignalP 3.0 Server suggests a high probability that there is an uncleavable signal peptide between residues 20 and 40 for both of the cotton apyrases (Bendtsen et al., 2004). AtAPY1 and AtAPY2 show 87% identity at the amino acid level and are more closely related to each other than the rest of the Arabidopsis apyrase gene family (Steinebrunner et al., 2000) and this might also be the case for GhAPY1 and GhAPY2. However it is possible that additional paralogous and homologous genes originating from progenitors will be identified after the cotton genome is sequenced. Because of the high degree of sequence similarity between GhAPY1 and GhAPY2 the inhibitors and antibodies used in this study most likely target both of these ectoapyrases.

Expression of *GhAPY1* and *GhAPY2* Correlates with Rapid Phase of Fiber Elongation

Approximately half of the ESTs for GhAPY1 and GhAPY2 were obtained from cotton fiber libraries, indicating that both apyrases are expressed during cotton fiber development. Quantitative reverse transcription (qRT)-PCR analysis performed using genespecific primers on epidermis (fiber) and ovule tissue over a time course during early fiber growth and development shows that GhAPY1 and GhAPY2 transcripts are both found in ovules and are enriched in growing cotton fibers (Fig. 1, A and B). The GhAPY1 mRNA was detected in fibers at 3 DPA, and its level is maintained at this high peak at 5 DPA. At 10 and 15 DPA *GhAPY1* mRNA levels are slightly lower than at 3 and 5 DPA but are still high. High message levels are found for GhAPY2 in 3-DPA fibers and these levels increase at 10 DPA and are maintained at this higher level at 15 DPA. GhAPY1 and GhAPY2 transcripts are also in leaf, stem, and petal tissue, but at relatively low levels, similar to the levels in 0-DPA ovule tissue (Supplemental Fig. S2). Immunoblot analysis using polyclonal anti-AtAPY1 antibodies indicated that a cross-reactive protein existed in 7-DPA fibers with a $M_{\rm r}$ slightly smaller than that of Arabidopsis AtAPY1 (Fig. 1C).

Apyrase Inhibitors and Anti-Apyrase Antibodies Inhibit Fiber Elongation

To test whether apyrase activity is important during cotton fiber growth we applied various concentrations of two different chemical apyrase inhibitors, apyrase inhibitor 4 and NGXT191, to cotton ovule cultures at 3 and 5 DPA. Both of these inhibitors are small, organic molecules that were selected from a chemical library based on a screen for specific inhibition of potato apyrase activity (Windsor et al., 2002). Application of all three concentrations of both inhibitors at 5 DPA resulted in statistically significant inhibition of fiber growth when cotton fibers were measured at 7 DPA (Fig. 2A). There was clearly a dose-dependent effect, and the effects at each dosage were statistically



Figure 1. *GhAPY1* and *GhAPY2* are expressed in cotton fibers during elongation phase of growth. A, Transcript abundance of *GhAPY1* is enhanced in fibers and is maintained at a high level during fiber growth. B, Transcript abundance of *GhAPY2* is enhanced in fibers and is upregulated during the later stages of fiber growth. Assays were done by qRT-PCR analysis using gene-specific primers, and the results were normalized to the level in ovules at 15 DPA (value of 1) for *GhAPY1* and to the level in ovules at 10 DPA (value of 1) for *GhAPY2*. Error bars represent sb. C, Immunoblot detection of an apyrase in cotton fibers isolated from 7-DPA cultured ovules. The 0-DPA lane shows lack of immunostain for 0-DPA whole ovules. Col-0 lane shows immunostain of apyrase from a crude extract of primary roots from liquid-grown seedlings of Arabidopsis (Col cultivar).

significant. Apyrase inhibitor treatments at 3 DPA also resulted in statistically significant inhibition of cotton fiber growth when fiber lengths were measured at 5 and 19 DPA (data not shown). The level of inhibition increased with increasing concentrations of both inhibitors. Treatment with pyridoxalphosphate-6azophenyl-2',4'-disulfonic acid (PPADS), an inhibitor of animal purinoceptors, reversed the inhibition of fiber growth caused by the apyrase inhibitors and returned fiber growth to control levels (Fig. 2B).

To determine if the observed growth effects were indeed due to inhibition of apyrase activity, we measured the amount of ATP found in the growth medium. The inhibitor treatments raised the amount of detectable ATP in the growth medium significantly above the control level of 330 nm (Fig. 2C), and increasing the amount of the inhibitor used resulted in higher levels of ATP measured. Application of the highest concentration of inhibitors NGXT191 and 4 caused a 2.1-fold and 3.2-fold increase in ATP levels, respectively.

We tested the effects of treating the cotton ovule cultures with polyclonal antibodies raised against Arabidopsis APY1 that had been demonstrated to inhibit apyrase activity (Wu et al., 2007). Treatment with immune sera led to statistically significant inhibition of fiber growth, while preimmune sera had no effect on growth (Fig. 3A). There was also a 3.3-fold and 5.3-fold increase in the level of detectable ATP after application of the lower and higher antibody concentrations, respectively (Fig. 3B).

Application of High Levels of ATP γ S and ADP β S Inhibit Fiber Elongation

The results from the inhibitor and antibody treatments suggested that accumulation of ATP in the fiber ECM causes inhibition of fiber elongation. To directly test this hypothesis, we applied poorly hydrolyzable nucleotides to ovule cell cultures. These nucleotides also induce signaling changes (Jeter et al., 2004), but are more resistant to hydrolysis by wall enzymes and are effective at lower concentrations than applied ATP and ADP (Roux et al., 2006). Application of 150 μ M ATP γ S or ADP β S at 5 DPA did indeed inhibit the growth of fibers when their length was measured at $\breve{7}$ DPA, and application of 150 μ M adenosine 5'-O-thiomonophosphate (AMPS), a closely related molecule but not a purinoceptor agonist, had no effect on growth (Fig. 4, A and B). There was also a statistically significant inhibition of cotton fiber growth at 5 DPA by 150 μ M ATP γ S when application was made at 3 DPA (data not shown). Furthermore, the effects of these high concentrations of ATP γ S and ADP β S were reversed by inclusion of PPADS and by adenosine.

Application of Low Levels of ATP γ S and ADP β S Promote Fiber Elongation

Representative images show that when the ovules were treated with 30 μ M ATP γ S at 5 DPA the cotton fibers grew noticeably longer by 7 DPA, and this promotion of growth could be blocked by the addition of PPADS and adenosine (Fig. 4A). Quantification of these growth changes indicated that they were statistically significant, and that ATP γ S and ADP β S at 30 μ M each induced an increase in average fiber length $\geq 44\%$ (Fig. 4C). We also observed statistically significant promotion of cotton fiber growth at 5 DPA by 30 μ M ATP γ S when application was made at 3 DPA, and a statistically significant increase in the growth rate between 5 and 7 DPA (data not shown). Application of either ATP γ S or ADP β S resulted in a biphasic doseresponse curve for fiber growth, with the threshold for promotion of growth somewhere between 10 and 30 μ M and the threshold for inhibition of growth somewhere between 125 and 150 μ M (Supplemental Fig. S3).

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Figure 2. Inhibition of apyrase activity in cotton ovule cultures using chemical inhibitors decreases overall fiber growth and increases eATP levels. A, Treatment of cotton ovule cultures with apyrase inhibitor NGXT 191 and apyrase inhibitor 4 at 3 and 5 DPA decreases average fiber lengths at 7 DPA. B, Inhibition of fiber growth induced by NGXT 191 and apyrase inhibitor 4 (5 μ g/mL) at 3 and 5 DPA is reversed by coincubation with 250 μ M PPADS. C, Treatment of cotton ovule cultures with NGXT 191 and apyrase inhibitor 4 at 3 and 5 DPA

Application of AMPS, which cannot activate purinoceptors in animal cells, had no effect on fiber growth. Neither 30 nor 150 μ M ATP γ S altered ovule size (data not shown).

Application of Ethylene Inhibitors Block the Growth-Stimulatory Effects of 30 μ M ATP γ S and the Growth-Inhibitory Effects of 150 μ M ATP γ S

The addition of 10 μ M aminovinylglycine (AVG) to the medium at 5 d after placing the ovules in culture (i.e. as the rapid elongation phase begins) did not alter the rate of cotton fiber growth (Supplemental Fig. S4A; Fig. 5A). However, coincubation of 10 μ M AVG with 30 μ M ATP γ S or with 150 μ M ATP γ S blocked the growth stimulatory or inhibitory effects of that concentration of nucleotide (Fig. 5A). The addition of 1 μ M silver nitrate (AgNO₃) to the medium at 5 d after placing the ovules in culture did not alter the rate of fiber growth (Supplemental Fig. S4B; Fig. 5B). However, coincubation of 1 μ M AgNO₃ with 30 μ M ATP γ S or with 150 μ M ATP γ S blocked the growth stimulatory or inhibitory effects of that concentration of nucleotide (Fig. 5B).

Application of the Ethylene Precursor, 1-Aminocyclopropane-1-Carboxylic Acid, Lowers the Concentration of ATP γ S Needed to Induce Growth Stimulation

As shown previously, application of 30 μ M ATP γ S at 5 DPA resulted in increased fiber growth at 7 DPA. The addition of 1 μ M 1-aminocyclopropane-1-carboxylic acid (ACC) or 10 μ M ATP γ S to the medium at 5 d after placing the ovules in culture did not alter the rate of fiber growth (Supplemental Figs. S3 and S4C; Fig. 5C). However, coincubation of 10 μ M ATP γ S and 1 μ M ACC induced increased cotton fiber growth (Fig. 5C).

Application of ATPγS Induces Increased Ethylene Production

We tested whether applied nucleotides induced ethylene production in three independent cotton ovule cultures. Over a 24 h period between 5 and 6 d after beginning the ovule culture, untreated cotton ovules showed relatively low ethylene production, the rate remaining below 2 pmol ethylene per ovule per hour during the entire period. Application of 150 μ M ATP γ S induced a statistically significant increase in ethylene production ($P \leq 0.006$), rising to more than 2.5-fold over the untreated control by 7 h after application, and this rate was significantly higher than the rate induced by 150 μ M AMPS at this time point (Supplemental

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increases the amount of ATP in the media surrounding the cotton fibers at 7 DPA. All values are the mean \pm sE from four biological replicates. Different letters above the bars indicate mean values that are significantly different from one another (P < 0.05; $n \ge 24$).



Figure 3. Inhibition of apyrase activity in cotton ovule cultures using apyrase antibodies decreases overall fiber growth and increases eATP levels. A, Treatment of cotton ovule cultures with polyclonal antiapyrase antibodies at 3 and 5 DPA decreases average fiber lengths at 7 DPA. The difference in growth of fibers treated with preimmune serum was not statistically different ($n \ge 24$); the difference in average fiber lengths treated with immune serum and treated with buffer is statistically significant ($P < 10^{-9}$; *n* in every case ≥ 20). The protein concentration of the preimmune sera was 0.3 $\mu g/\mu L$, and of the immune sera was 0.46 $\mu g/\mu L$. B, Treatment of cotton ovule cultures with polyclonal anti-apyrase antibodies at 3 and 5 DPA increases the amount of ATP surrounding the cotton fibers at 7 DPA. All values are the mean \pm st from four biological replicates. Different letters above the bars indicate mean values that are significantly different from one another (P < 0.05; $n \ge 24$).

Table S1). On the other hand the rates of ethylene production induced by 30 μ M ATP γ S or AMPS were not significantly different from that induced by the control (P > 0.05; Supplemental Table S1). When measured 48 h after nucleotide application, fiber lengths of ovules treated by 150 μ M ATP γ S were significantly shorter than controls, and fibers of ovules treated by 30 μ M ATP γ S were significantly longer than controls. Because $ATP\gamma S$ has been reported to inhibit apyrase activity in animals in certain cases, we measured the levels of ATP in the media after treatment with $ATP\gamma S$. Although this treatment did increase media ATP levels, these increases were small compared to the concentration of the applied $ATP\gamma S$ (data not shown), indicating the growth changes are due to $ATP\gamma S$ treatment and not inhibition of apyrase activity.

DISCUSSION

Ectoapyrases appear to be important growthregulating proteins that are evolutionarily conserved in plants (Clark and Roux, 2009). Genetic and biochemical approaches have been used to show that two ectoapyrases in Arabidopsis, AtAPY1 and AtAPY2, are critically needed for growth. For example, Arabidopsis double-knockout mutants (*apy1apy2*) are severe dwarfs, and inhibition of ectoapyrase activity with either chemical inhibitors or specific antibodies leads to inhibition of growth (Wolf et al., 2007; Wu et al., 2007). In addition, the expression of these two apyrases is highest in actively growing tissues (Wolf et al., 2007; Wu et al., 2007). Consistent with this result, higher levels of eATP are found in the ECM of actively growing tissues in Medicago truncatula (Kim et al., 2006). This correlation of apyrase expression and localization of eATP in growing cells suggests that it is important to regulate the eATP signal during growth. The fact that the cotton fiber apyrases *GhAPY1* and *GhAPY2*, which both have high sequence similarity to AtAPY1 and AtAPY2, are up-regulated during the rapid phase of fiber elongation further highlights the apyrase-growth connection. Moreover, although these apyrases are expressed in stems, leaves, and petals, their expression is clearly enhanced in the more rapidly growing fiber tissue.

Motif analysis indicates that both GhAPY1 and GhAPY2 contain a putative uncleavable signal peptide located near their N terminus. This structure could anchor both apyrases to the membrane at their N terminus and orient the protein domains responsible for enzyme activity out into the ECM, allowing these enzymes to function as ectoapyrases, like their close homologs AtAPY1 and AtAPY2. Either or both of these cotton apyrases may also be a secreted protein, as is the case for the ectoapyrases in Arabidopsis pollen (Wu et al., 2007), however we were unable to detect any apyrase activity free in the media (data not shown). Consistent with the interpretation that the cotton apyrases are ectoapyrases, suppression of fiber apyrase activity by two selective inhibitors and specific anti-apyrase antibodies leads to an increase in the concentration of the eATP associated with growing cotton fibers. The two chemical apyrase inhibitors have been well characterized (Windsor et al., 2002, 2003), and have been shown to inhibit pollen tube germination and elongation (Wu et al., 2007). They also inhibit fiber elongation in parallel with the rise in [eATP].

Although most of the fibers we measured were not fully submerged, we found that up to 25% of the length of most of the fibers was in direct contact with the media and additives. We also observed wicking of the media over approximately half of the fiber lengths. A recent report indicates that growing cotton fibers are connected by an outer layer of the cell wall and this might be expected to facilitate the wicking effect (Singh et al., 2009). Additionally, all of the treatment

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Figure 4. A total of 150 μ M ATP γ S or ADP β S decrease overall cotton fiber growth and 30 μ M ATP γ S or ADP β S increase overall cotton fiber growth. A, Representative image of effects of treatment with 150 μ M ATP γ S alone or 30 μ M ATP γ S alone, or these treatments coincubated with 250 μ M PPADS or 50 μ M adenosine on cotton fiber growth. Bar = 1 mm. B, Application of 150 μ M ATP γ S or ADP β S at 5 DPA to cotton ovule cultures decreases average fiber lengths at 7 DPA and this decrease is reversed by coincubation with 250 μ M PPADS or 50 μ M adenosine. AMPS was tested at 150 μ M and had no effect on growth. C, Application of 30 μ M ATP γ S or ADP β S at 5 DPA to cotton ovule cultures increases average fiber lengths at 7 DPA, and this increase is reversed by coincubation with 250 μ M PPADS or 50 μ M adenosine. All values are the mean ± st from four biological replicates. Different letters above the bars indicate mean values that are significantly different from one another (P < 0.05; $n \ge 24$).

chemicals except antibodies would be easily small enough to enter the wall space of the fiber and diffuse further up toward the tip of the fiber through the apoplasm, or wick up toward the tip along the fiber surface. Although antibodies can penetrate into wall spaces (Cosgrove, 1999), they may not diffuse as efficiently or as far as the smaller agents tested. Nonetheless they would still be in direct contact with part of the fiber ECM, and thus could inhibit a certain fraction of the ectoapyrase activity in the fiber.

Both of the apyrase inhibitors are sufficiently hydrophobic to cross the plasma membrane and target apyrases inside the cell, and this could also affect fiber growth. However the chemical inhibitor effects are replicated by anti-apyrase antibodies, which are unlikely to cross the plasma membrane. Thus the finding that the antibody treatment also resulted in inhibition of fiber growth and accumulation of eATP strongly supports the hypothesis that ectoapyrase activity (i.e. activity that would hydrolyze eATP) is critical for fiber elongation. The antibodies and apyrase inhibitors would likely inhibit any ectoapyrase expressed in cotton fibers, and so, although *GhAPY1* and *GhAPY2* expression correlates closely with fiber growth, they may not be the only ectoapyrases that regulate cotton fiber growth.

Although it takes >100 μ M ATP γ S to inhibit fiber growth, when apyrase inhibitors inhibit fiber growth the change observed in media ATP concentration is less than 2 μ M. Why this discrepancy? For signaling purposes the key [eATP] is the concentration nearest the receptor at the surface of the plasma membrane. Because ATP released at the cell surface of the fibers would be both diluted and hydrolyzed as it diffuses into the bulk medium of the ovule culture, the changes we observe in [eATP] in the bulk medium after apyrase inhibition would be expected to be a small fraction of the changes at the receptor binding site. Similarly, because the media-applied nucleotides would be diluted, bound to other proteins in the ECM (e.g. kinases, phosphatases, and apyrases), and perhaps also biochemically modified as it diffuses in to the cell surface, their concentration would be expected to be significantly lower by the time they actually reach the receptor binding site. Nonetheless, these considerations may only partially explain why apyrase inhibition does not result in a greater change in the media [ATP]. Schicker et al. (2009) report that



Figure 5. Agents that alter ethylene production or activity modify the effects of applied nucleotides on fiber growth. A, Increased cotton fiber growth induced by 30 μ M ATP γ S and decreased cotton fiber growth induced by 150 μ M ATP γ S are reversed by AVG. The difference in growth of fibers incubated with 10 μ M AVG alone was not statistically different ($n \ge 24$). B, Increased cotton fiber growth induced by 30 μ M ATP γ S and decreased cotton fiber growth induced by 150 μ M ATP γ S are reversed by AgNO₃. The difference in growth of fibers incubated with 1 μ M AgNO₃ alone was not statistically different ($n \ge 24$). C, Application of ACC at 5 DPA lowers the concentration of ATP_γS needed to induce increased fiber elongation, measured at 7 DPA, from 30 to 10 μ M. The difference in growth of fibers treated with 10 μ M ATP γ S or 1 μ M ACC alone was not statistically different ($n \ge 24$). All values are the mean \pm sE from four biological replicates. In all sections, different letters above the bars indicate mean values that are significantly different from one another (P < 0.05; $n \ge 24$).

in animals some ectoapyrase enzymes form heterocomplexes with P2Y purinoceptors. If this is true also in plants, and plant apyrases influence receptor function or sensitivity, then apyrase inhibition could alter the ATP signaling pathway more directly, and not just by altering the [eATP]. There is another connection between apyrase inhibition and purinoceptor function. The ATP γ S agonist, in addition to activating purinoceptors, also has the potential to bind to the active site of apyrases and antagonize their activity. In animals ATP γ S is typically not a strong antagonist of apyrase activity (Yegutkin and Burnstock, 2000), and this appears to be true also in cotton fibers.

Thus, in the ovule culture system, the effects of ATP γ S would more likely be attributable to its ability to stimulate a plasma membrane-localized purinoceptor (Demidchik et al., 2009) than to its ability to antagonize apyrase activity. These considerations lead us to predict that the metabolic pathway that links eATP to growth changes in cotton begins in plants, as in animals, with the binding of eATP to a plasma membrane receptor. Thus far the plant eATP receptor has not been found, but recently eATP receptors have been identified in the slime mold *Dictyostelium* and in green algae (Fountain et al., 2007, 2008). There does appear to be some similarity pharmacologically between animal and plant eATP receptors, because an antagonist to animal cell purinoceptors, PPADS, also blocks eATP-dependent responses in plant cells. However, as yet there is no confirmation of its action on a biochemical level.

Adenosine is one of the final products of eATP turnover and as such could serve as negative feedback molecule. In animal cells it is well established that adenosine participates in a diverse array of responses via a family of adenosine P1 receptors that are distinct from P2 receptors (Cronstein et al., 1985; Bengtsson et al., 1996). Adenosine can also function as a negative regulator of neurotransmitter release in animal cells, functioning together with ATP to modulate smooth muscle contraction (Burnstock, 1996). Adenosine blocked the ability of eATP to induce superoxide production and gene expression changes in Arabidopsis leaves (Song et al., 2006), and it also inhibited eATPinduced wound responses in algal cells (Torres et al., 2008). Here we observed that adenosine could also block eATP-induced changes in fiber elongation, revealing that its ability to act as an antagonist in eATPinduced plant responses extends to growth responses. In plants to date there are no reports of extracellular adenosine receptors, so the mechanism by which adenosine exerts its effects in plant cells is unclear. However, the signaling steps downstream of eATP in plants include O_2^- production, so it may be that adenosine inhibits O_2^- production in plants as it does in animals, which results in adenosine acting as an antagonist in plant eATP responses.

Recent results in cotton implicate ethylene as a key mediator of cotton fiber growth (Shi et al., 2006; Qin

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et al., 2007), and in Arabidopsis eATP treatment induces an increase in the transcript level of ACC synthase, the rate-limiting enzyme in ethylene biosynthesis, as well as several ethylene-responsive factors (Jeter et al., 2004; Song et al., 2006). Given the known role of ethylene in cotton fiber growth and the upregulation of genes in the ethylene signaling pathway by eATP, this raised the question of whether changes in cotton fiber growth in response to application of nucleotides were dependent on ethylene production.

In Shi et al. (2006), 1 μ M AVG can completely suppress cotton fiber growth in cotton ovules if it is applied before fiber initiation. However, if it is applied at day 5 after fiber initiation, 10 μ M AVG does not inhibit fiber growth, but it does block the ability of 30 μ M ATP γ S to stimulate fiber elongation as well as the ability of 150 μ M ATP γ S to inhibit fiber elongation. Thus far only ethylene-induced promotion of fiber growth has been reported, however it is known that in many plant tissues ethylene can both inhibit and promote growth depending on its concentration (Pierik et al., 2006). The ability of AVG to block inhibition of fiber growth induced by high levels of ATP γ S and the ability of higher concentrations of ACC to also inhibit fiber growth is novel evidence that ethylene exhibits a biphasic dose-response curve on cotton fiber growth.

To further evaluate the AVG results, we tested the ability of another ethylene antagonist, AgNO₃, to block changes in fiber growth induced by application of nucleotides. Our finding that AgNO₃ could prevent both the promotion and inhibition of fiber growth induced by applied nucleotides were in agreement with and supported the AVG data. Taken together, these results are consistent with the interpretation that both the stimulation and the inhibition of fiber growth by applied nucleotides require the mediation of ethylene. However, it is unlikely that ethylene would be the only hormone influencing the growth effects. There is well-documented cross talk between gibberellin, auxin, and ethylene (Smalle et al., 1997; Saibo et al., 2003; Vandenbussche et al., 2003). The ethylene effects we observed occurred in the presence of both GA₃ and auxin, and may have been influenced by them, since both are required for initiation of fiber development in the ovule culture system.

Consistent with there being a role for ethylene in mediating the fiber growth effects of applied nucleotides, we found that the high doses of ATP γ S that inhibit cotton fiber growth also induce a significantly increased rate of ethylene production by 7 h after treatment. Because inhibition of ectoapyrase activity by antibody or inhibitor treatment causes an increase in naturally occurring levels of eATP/eADP and mimics the inhibition of growth induced by treatment with high levels of ATP γ S, ethylene production would be a likely downstream result of inhibiting ectoapyrase activity in cotton fibers. However, AMPS, which does not affect fiber growth, also induced a significant increase in ethylene production. Although this increase was clearly lower than that induced by ATP γ S, this result and the growth data, taken together, indicate that although ethylene induction is likely a key step in the signaling pathway linking eATP to fiber growth changes, an increase in ethylene alone is not sufficient to mediate these growth changes.

The fact that 10 μ M AVG blocks the ability of 30 μ M ATP γ S to promote fiber growth underscores the need for ethylene production in this response. Also pointing to a role for ethylene in mediating the growth-promotive effects of low [ATP γ S] is the finding that 10 μ M ATP γ S, which alone cannot promote growth, can suffice to promote fiber growth when 0.1 μ M ACC is in the medium, even though 0.1 μ M ACC (nor any other concentration we tested) could alone promote fiber growth. Others also have observed that trying to use ACC alone to induce ethylene levels to the low concentrations needed to observe promotion of growth in plant tissues is problematic (Pierik et al., 2006).

Although ethylene production appears to be needed for low concentrations of ATP γ S to promote fiber growth, the rate of ethylene production induced by 30 μ M ATP γ S was not statistically different from that induced in the control and by 30 μ M AMPS when measured 7 h after treatment. If 30 μ M ATP γ S induces increased ethylene production, this increase could be transient and could happen at any time point after nucleotide treatment. Clearly, more measurements at more time points would be needed to resolve whether the stimulation of growth by low [ATP γ S] is mediated in part by increased ethylene production.

Using qRT-PCR analysis we found that 1 h after treatment of 5 DPA cultures with 150 μ M ATP γ S there was a 50% and 70% increase in message levels for *ACO1* and *ACO4*, respectively, while message levels for *ACO2* and *ACO3* and *ACS6* and *ACS7* were unchanged (data not shown). Thus, the increase in the concentration of ethylene induced by treatment with 150 μ M ATP γ S may be due to an increase in the activity of two *ACOs*. These results support the suggestion made by Qin et al. (2007) that ethylene biosynthesis during cotton fiber elongation may be regulated at the level of ACO activity and not at the level of ACS activity, which is often the rate-limiting step for ethylene production in other tissues.

The effects of eATP in Arabidopsis are transduced by an initial increase in the concentration of cytosolic calcium, $[Ca^{2+}]_{cyt}$ (Demidchik et al., 2003; Jeter et al., 2004). In many plant tissues downstream of this eATPinduced calcium signal there is an increase in the production of reactive oxygen species and nitric oxide (Kim et al., 2006; Song et al., 2006; Foresi et al., 2007; Cardenas et al., 2008; Torres et al., 2008; Wu and Wu, 2008; Demidchik et al., 2009; Reichler et al., 2009). Both of these signaling agents appear to play an important role during plant growth (Foreman et al., 2003; Prado et al., 2004). There are hormone-mediated signaling pathways that could also be interacting with the eATP signal to regulate fiber growth. For example, apyrases and eATP have been implicated in the inhibition of the transport of the growth hormone, auxin (Tang et al.,

2003). Additional studies will be needed to ascertain whether one or more of these other signaling agents play a role along with ethylene in eATP-mediated growth changes in cotton fibers.

Our cotton fiber results parallel earlier findings in pollen tubes, where eATP/apyrase signaling also regulates growth (Wu et al., 2007). Although cotton fibers appear to grow more via diffuse growth (Tiwari and Wilkins, 1995) and pollen tubes grow via polar tip growth (McKenna et al., 2009), in both cell types growth would likely be accompanied by the delivery of secretory vesicles to the plasma membrane at expansion sites and the release of some ATP there, as occurs during the polar growth of root hairs (Kim et al., 2006). The coordinated increase of GhAPY1 and *GhAPY2* expression during the rapid growth phase of fibers may be needed to maintain the [eATP] at levels optimal for growth. Genetic studies will be needed to further clarify the precise functions of these two ectoapyrases and the role of extracellular nucleotide signaling during cotton fiber growth.

MATERIALS AND METHODS

Plant Materials

Upland cotton (*Gossypium hirsutum*; TM-1) was grown in 5-gallon pots using Metro Mix 700 (Sun Gro Horticulture) soil mixture in a temperaturecontrolled greenhouse (90°F day and 75°F night). Two-week-old seedlings were treated with Osmocote 14-14-14 (The Scotts Co.) and then fertilized once per week with a 1:10 diluted solution of Peters 20-20-20 (Peters Professional). Flowers were harvested for in vitro ovule culture experiments. Ovules were harvested at various DPA time points and frozen using liquid nitrogen (LN) for RNA extraction.

Sequence Analysis of GhAPY1 and GhAPY2

Cotton ESTs DT562985 and DT564579, generously supplied by A. Woodward in the Z.J. Chen lab, were used to assemble a putative GhAPY1 full-length consensus sequence and EST TC150263 was used as a putative GhAPY2 full-length sequence. These sequences were used to design PCR primers for GhAPY1 and for GhAPY2 (Supplemental Table S2). Cotton leaf RNA was isolated using the Sigma spectrum plant total RNA kit, and first-strand CDNA was synthesized and used as template for Roche Expand Hi-Fidelity Taq DNA polymerase to amplify cotton apyrases. For GhAPY1, amplified message was cloned into the expression vector pTrcHis-2 using Invitrogen pTrcHis and pTrcHis-2 TOPO TA expression kit. GhAPY1 clones and GhAPY2 amplified message were sequenced by the University of Texas Core DNA facility with ABI 3730 DNA analyzer. For the GhAPY2 full-length sequence, the majority of the sequence is based on direct sequencing of the PCR product but the 48 bp at the N terminus and the 49 bp at the C terminus are based on EST data.

RNA Extraction from Cotton Ovules and Fibers

For assay of *GhAPY1* and *GhAPY2* mRNA levels, ovules and fibers were dissected from the cotton bolls collected at -3 (3 d prior to anthesis), 0 (on the day of anthesis), +3, +5, +10, and +15 DPA. To separate the ovules from fibers, the ovules with fiber cells at 3, 5, 10, and 15 DPA were frozen immediately in LN with or without the glass beads as described by Taliercio and Boykin (2007). The frozen tissues were gently swirled using a pestle in a mortar with LN until the fibers were separated from ovules. The solution in LN was filtered through a 500- μ m nylon mesh (Small Parts, Inc.) so that the ovules remained at the top of mesh and the fibers went through with LN. The separated ovules or fibers were ground in LN. The ground tissues were subjected to RNA extraction using the method described by Lee et al. (2006)

and the Spectrum plant RNA kit (Sigma-Aldrich). For assay of *ACO1-4* and *ACS6-7* mRNA levels, ovules with fibers were isolated from 5 DPA cultures 1 h after treatment with 150 μ M ATP γ S. Whole ovules were frozen in LN and then total RNA was extracted using the Invitrogen plant RNA purification reagent (Invitrogen).

qRT-PCR Analysis

First-strand cDNA synthesis was done using total RNA and SuperScript III (Invitrogen). For the transcript amplification, GhAPY1 gene-specific primers and GhAPY2 gene-specific primers (Supplemental Table S2) were designed using Primer Express version 2.0 software (Applied Biosystems) where the reverse primers were based on 3'-untranslated region sequences. Primers for ACO1-4 and ACS6-7 were designed based on the primers used by Qin et al. (2007). The qRT-PCR reaction was carried out in a final volume of 20 μ L containing 10 μL SYBR Green PCR master mix, 1 $\mu {\rm M}$ forward and reverse primers, and 0.1 µM cDNA probe in a ABI7500 real-time PCR system (Applied Biosystems). Cotton HISTONE3 (AF024716) was used to normalize the amount of gene-specific RT-PCR products (Wang et al., 2004). All reactions were performed in three biological replications and nine technical replications and the data were analyzed using ABI7500 SDS software (version 1.2.2). Fold changes for GhAPY1 and GhAPY2 were calculated using HISTONE3 as a control. Relative expression levels were calculated in reference to 15-DPA ovule values for GhAPY1 and 10-DPA ovule values for GhAPY2. Fold changes for ACO1-4 and ACS6-7 were calculated in reference to untreated control 5-DPA ovule values.

Immunoblot Analysis

At 0 and 7 DPA of in vitro ovule culture, cotton ovules were harvested. Arabidopsis (Arabidopsis thaliana) primary root tissue (Columbia-0 [Col-0]) was harvested after 8.5 d of liquid growth in 1.5% Suc/0.5 Murashige and Skoog media. Samples were frozen with LN and then ground at 4°C using a micro mortar in the presence of a buffer containing 45 mM Tris, 150 mM NaCl, 1.2 mm EGTA, 5 mm dithiothreitol, and SIGMAFAST protease inhibitor cocktail (Sigma). Samples were boiled in SDS sample buffer for 3 min and then centrifuged for 30 s at room temperature. Proteins were then separated via 10% SDS-PAGE, transferred to 0.45 μ m nitrocellulose membranes (Schleicher & Schuell), and blocked for 2 h with 1% dry milk in PBS pH 7.5 (Blotto). The membrane was then incubated overnight at 4°C with protein A-sepharose purified anti-Apy1 antibody diluted 1:250 with 1% Blotto. After three washes in 1% Blotto, the membrane was incubated for 1 h at room temperature with affinity-purified goat anti-guinea pig IgG coupled to an 800-nm fluorochrome diluted 1:10,000 (IRDye 800CW; Rockland Immunochemicals). After three washes with Blotto, the fluorochrome signals were detected and analyzed using the Odyssey infrared imaging system (LI-COR Biosciences).

In Vitro Cotton Ovule Culture

Cotton bolls were harvested and used within 24 h of flower opening and all petals bracts, and sepals were removed. If the bolls were not used immediately they were stored at 4°C until ready for use. The following procedures were taken from Cotton Fiber Bioscience Web site (http://msa.ars.usda.gov/la/ srrc/fb/oc.html). For sterilization, bolls were immersed into an 85% ethanol solution for at least 30 s but not more than 2 min. The excess ethanol was shaken off and the boll was passed through a flame until the residual ethanol was burned off. Ovules were then excised and placed in 10 mL of BT cotton media cell culture (Caisson Laboratories) + 5.0 μ M indoleacetic acid and 0.5 μ M GA_3 (Sigma-Aldrich), in 60 \times 15 mm petri dishes, and were then wrapped in MilliWrap (Millipore Corporation). BT cotton media cell culture contained: 0.272 g/L K₂HPO₄, 0.372 g/L CaCl₂, 0.493 g/L MgSO₄, 0.017 g/L MnSO₄, 0.009 g/L ZnSO4, 5.055 g/L KNO3, 0.008 g/L FeSO4 7H2O, 0.011 g/L EDTA-Na2/ 0.008 g/L BTM vitamin stock, and 0.18 g/L myoinositol. Ten to 12 cotton ovules were placed into each petri dish. Ovules from different flowers were randomized in each dish. Ovules were cultured at 32°C in the dark without agitation. Ovules were placed in a 75% ethanol solution for 15 min and then taken out and combed. Fiber lengths were measured manually with a dissecting microscope after combing the fiber cells away from the ovule with forceps and dissecting probe. Statistical analysis of fiber lengths was done using the Student's t test.

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In Vitro Cotton Ovule Culture Treatments

Stock solutions for apyrase inhibitors (NGXT191 and AI#4) were prepared in dimethyl sulfoxide at 2.5 mg/mL. Stock solutions for ATP γ S, ADP β S, AMP, PPADS, adenosine, (S)-trans-2-amino-4-(2-aminoethoxy)-3-butenoic acid hydrochloride (AVG), and ACC hydrochloride (Sigma-Aldrich) were prepared in deionized water at 50 mM. AgNO₃ (Sigma-Aldrich) was prepared in deionized water at 10 mM. All these stocks were stored at -20° C while not in use. For experiments testing the effects of apyrase inhibitors on cotton fiber growth, apyrase inhibitors were applied to cotton media cell culture at 3 and then again at 5 DPA after the introduction of cotton ovules. For control cultures the same concentration of dimethyl sulfoxide used for the cultures treated with apyrase inhibitors was added at both 3 and 5 DPA. For experiments testing the effects of apyrase antibodies on cotton fiber growth, the antibodies were applied to cotton media cell culture at 3 and 5 DPA. For experiments testing the effects of apyrase antibodies on cotton fiber growth, the antibodies were applied to cotton media cell culture at 3 and 5 DPA after the introduction of cotton ovules.

For experiments testing the effects of applying nucleotides during cotton fiber growth, various concentrations of ATP γ S, ADP β S, and AMP were added to cotton media cell culture at 3 DPA for experiments measuring fiber lengths at 5 DPA and at 5 DPA for experiments measuring fiber lengths at 7 DPA. For experiments testing the effects of PPADS and adenosine, these compounds were applied at the appropriate concentration to cotton media cell culture at 3 and 5 DPA for inhibitor and antibody experiments and 5 DPA for applied nucleotide experiments. For experiments testing the effects of AVG, ACC, and AgNO₃, various concentrations of these compounds were applied to cotton media cell culture at 5 DPA.

The production of the anti-AtAPY1 antibodies used is described by Steinebrunner et al. (2003). The crude immune and preimmune sera were purified using protein A-sepharose following the protocol described by Martin (1982), except that the buffers used were azide free. Bio-rad assay was used to determine that the concentration of the immune and preimmune sera that were 0.46 μ g/mL and 0.3 μ g/mL, respectively.

Assay of ATP Level in Cotton Ovule Media

For experiments measuring the [ATP] of the cotton ovule medium, aliquots (30 µL) of media were removed from incubating cotton ovule cultures at either 2, 5, or 7 DPA and immediately frozen in LN until they were analyzed for ATP concentration, as described by Wu et al. (2007). To collect media at the periphery of the ovule, ovules were removed from the media with forceps, clasping only the dry cotton fiber, and placed on a clear plastic surface. Then samples of media (typically no more that $60 \ \mu$ L) were drawn using a pipette. The [ATP] of cotton ovule media in which cotton ovules were growing was measured as described by Wu et al. (2007). Three individual 10 μ L samples were assayed from each sample to ensure internal consistency of the sample. Because ATP γ S has been reported to be an adenylyl donor for the formation of the luciferase-luciferin-AMP complex (Ortiz et al., 1993), when we determined the [ATP] in the medium of cotton ovule cultures treated with ATP_yS, measurements were made for both cultures with and without ovules. The [ATP] measured for cultures without ovules was subtracted from the [ATP] measured for cultures with ovules to accurately determine the [eATP] in the medium of cotton ovule cultures in the presence of 30 and 150 µM ATP_γS, respectively.

Ethylene Measurements

Fourteen ovules were cultured in petri dishes as described above. On day 6, the treatments were added to the media and 3 h later, lids fitted with rubber septa were sealed to the dish bottoms with several layers of Parafilm. After 4 h of incubation, 1 mL headspace samples were removed and analyzed on a Photovac 10SPlus GC equipped with a Carbopak B HT column and a photoionization detector. Ethylene levels were quantified by comparing the peak areas of experimental samples to those of known quantities of ethylene standards. Statistical analysis of ethylene production was done using ANOVA.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AF093604 (AtAPY1), AF141671 (AtAPY2), GU385147 (GhAPY1), and GU385148 (GhAPY2).

Supplemental Data

The following materials are available in the online version of this article.

- Supplemental Figure S1. Alignment performed with ClustalW2 of the deduced amino acid sequences of AtAPY1, AtAPY2, GhAPY1, and GhAPY2. The four characteristic apyrase conserved regions are boxed.
- Supplemental Figure S2. *GhAPY1* and *GhAPY2* are expressed in a variety of cotton tissues.
- Supplemental Figure S3. Biphasic dose-response curve for ATP γ S and ADP β S.
- **Supplemental Figure S4.** Dose-response cotton fiber growth curves for AVG, AgNO₃, and ACC.
- Supplemental Table S1. Treatment averages are for at least three replicates and measurements of ethylene production in pMoles ethylene/ovule/h made 7 h after treatment.

Supplemental Table S2. Primers used in this study.

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