GhPIPLC2D promotes cotton fiber elongation by enhancing ethylene biosynthesis

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HIGHLIGHTS
- GhPIPLC2D positively regulates cotton fiber elongation
- GhPIPLC2D cleaves PIP2 into IP3, which could be phosphorylated to IP6
- IP6 enhances fiber elongation via improving ethylene biosynthesis

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GhPIPLC2D promotes cotton fiber elongation by enhancing ethylene biosynthesis

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SUMMARY
Inositol-1,4,5-trisphosphate (IP₃) is an important second messenger and one of the products of phosphoinositide-specific phospholipase C (PIPLC)-mediated phosphatidylinositol (4,5) bisphosphate (PIP₂) hydrolysis. However, the function of IP₃ in cotton is unknown. Here, we characterized the function of GhPIPLC2D in cotton fiber elongation. GhPIPLC2D was preferentially expressed in elongating fibers. Suppression of GhPIPLC2D transcripts resulted in shorter fibers and decreased IP₃ accumulation and ethylene biosynthesis. Exogenous application of linolenic acid (C₁₈:₃) and phosphatidylinositol (PI), the precursor of IP₃, improved IP₃ and myo-inositol-1,2,3,4,5,6-hexakisphosphate (IP₆) accumulation, as well as ethylene biosynthesis. Moreover, fiber length in GhPIPLC2D-silenced plant was reduced after exogenous application of IP₆ and ethylene. These results indicate that GhPIPLC2D positively regulates fiber elongation and IP₃ promotes fiber elongation by enhancing ethylene biosynthesis. Our study broadens our understanding of the function of IP₃ in cotton fiber elongation and highlights the possibility of cultivating better cotton varieties by manipulating GhPIPLC2D in the future.

INTRODUCTION
Inositol-1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG) are two important second messengers that convert extracellular signals to intracellular signals in plants (Singh et al., 2015). Phosphoinositide-specific phospholipase C (PIPLC) catalysis of the substrate phosphatidylinositol (4,5) bisphosphate (PIP₂) produces both messenger molecules (Abd-El-Haliem and Joosten, 2017). Reversible inactivation of guard cell K⁺ channels is controlled by cytoplasmic Ca²⁺ that rely on IP₃ signal cascades (Blatt et al., 1990). In tomato plants, reduction of IP₃ content modifies the inositol phosphate pathway and affects light signaling and secondary metabolism (Alimohammadi et al., 2012). IP₃ suppresses protein degradation in plant vacuoles by regulating sorting nexin-mediated protein sorting (Chu et al., 2016). In post-harvest peach fruit, IP₃ is also involved in nitric oxide-enhanced chilling tolerance and defense response (Jiao et al., 2019).

When phosphorylated, IP₃ forms inositol hexaphosphate (Dong et al., 2019), which has many functions in plants. Also known as phytic acid, IP₆ is the main form of storage of phosphorus in mature seeds (Gibson et al., 2018). Inositol hexaphosphate can stimulate Ca²⁺ release to participate in many signaling pathways (Lee et al., 2015). As an endomembrane-acting Ca²⁺ release signal, IP₆ activates both fast and slow conductance of the guard cell vacuole (Lemtiri-Chlieh et al., 2003). In plant hormone perception, IP₆ can bind to the auxin receptor complex TIR1/IAA (Tan et al., 2007). Gibberellic acid has been shown to affect the degradation of IP₆ in soybean sprouts with the calcium transport (Hui et al., 2018).

Phosphatidylinositol (PI), the precursor of IP₃, is composed of 1,2-DAG phosphate and inositol. As the major phospholipid in cell membranes, PI plays critical roles in various physiological processes in plants (Hänninen et al., 2017; Heilmann, 2016). Phosphorylation of PI produces phosphatidylinositol 4-phosphate (PIP₁) of which can be further catalyzed to generate PIP₂ (Munnik and Nielsen, 2011), a kind of membrane phospholipid involved in various developmental stages in plants (Shimada et al., 2019; Kusano et al., 2008). Mitogen-activated protein kinase 6 (MPK6)-mediated phosphorylation of PI 4-phosphate 5-kinase 6 limits the production of the pool of functional PIP₂ in response to the pathogen-associated molecular pattern-triggered immunity in Arabidopsis thaliana (Menzel et al., 2019). Directional growth is regulated by...
PIPLC, an important lipid hydrolase in plants, cleaves PIP2 into two important secondary messengers, IP3 and DAG (Mueller-Roeber and Pical, 2002; Kadamiur and Ross, 2013). The four conserved domains of PIPLC are named EF-hand, PI-PLC-X, PI-PLC-Y, and C2 (Zhang et al., 2018a). The EF-hand domain consists of two helix-loop-helix folding motifs for calcium-binding. The catalytic activity of all PIPLCs is strictly dependent on the PI-PLC-X and PI-PLC-Y domains. The C2 domain has been identified in all plant PIPLCs and functions along with the participation of calcium, in binding phospholipids (Pokotylo et al., 2014). The PIPLC plays multiple roles in plant stress response and development.

There are nine AtPIPLC genes in Arabidopsis (Tasma et al., 2008). AtPIPLC2 is required for seedling growth (Di Fino et al., 2017) and AtPIPLC5 is involved in primary and secondary root growth (Zhang et al., 2018c). AtPIPLC3 and AtPIPLC9 play critical roles in thermo-tolerance response (Gao et al., 2014; Zheng et al., 2012). AtPIPLC4 is up-regulated after salt stimulation (Tasma et al., 2008). In addition, overexpression of AtPIPLC5 and AtPIPLC7 improves plant drought tolerance (Zhang et al., 2018c; Van Wijk et al., 2018). AtPIPLC2 silenced plants are more susceptible to bacterial and fungal infections, suggesting that AtPIPLC2 is involved in plant immune response (D’Ambrosio et al., 2017).

Cotton fiber is an important industrial textile material in the world (Li et al., 2015). Fuzz and lint are two types of cotton fibers. Fuzz fibers only grow to a maximum length of 5 mm after seed maturity which cannot be used in textile (Arpat et al., 2004). Lint fibers develop into sufficiently long fibers desired for textile products (Kim and Tripplett, 2001). The fuzzless and lintness mutant (fl) has been widely used to investigate the developmental mechanism of cotton fibers (Wu et al., 2017; Hu et al., 2018). Multiple genes are reported to be involved in cotton fiber development, including genes related to phytohormones (Xiao et al., 2019; Zhang et al., 2011), plant growth and development (Zhang et al., 2018a), and biotic and abiotic stress responses (Ile et al., 2019). Linolenic acid (C18:3) enhanced cotton fiber elongation by improving PI and phosphatidylinositol monophosphate growth and development (Zhang et al., 2018a), and biotic and abiotic stress responses (He et al., 2019). Linolenic acid (C18:3) enhanced cotton fiber elongation by improving PI and phosphatidylinositol monophosphate growth and development (Zhang et al., 2018a), and biotic and abiotic stress responses (He et al., 2019). Linolenic acid (C18:3) enhanced cotton fiber elongation by improving PI and phosphatidylinositol monophosphate growth and development (Zhang et al., 2018a), and biotic and abiotic stress responses (He et al., 2019).

Ethylene, one of the major hormones in plants, participates in cotton fiber development (Li et al., 2007; Qin et al., 2007; Shi et al., 2003, 2006). The transcripts of three ethylene biosynthesis genes 1-aminocyclopropane-1-carboxylic acid oxidases (GhACO1-3) were highly accumulated at the fiber elongation stage. Exogenous application of ethylene promotes fiber elongation, as evidenced by in vitro application of an ethylene-synthesis inhibitor, L-(2-aminoethoxyvinyl)-glycine, that hindered cotton fiber elongation (Shi et al., 2003, 2006). Ethylene may also promote fiber elongation by enhancing H2O2 production, which in turn induces ascorbate peroxidase activity (GhAPX1) in cotton fibers. The high expression of GhAPX1 observed in wild-type (WT) cotton fibers and little to no expression of GhAPX1 observed in fuzzless-lintness (fl) mutant ovules suggest an important role of GhAPX1 in fiber development (Li et al., 2007). Lignoceric acid can also enhance fiber cell elongation by increasing ethylene biosynthesis. Moreover, ethylene can eliminate the inhibition of fiber cell elongation caused by application of 2-chloro-N-[ethoxymethyl]-N-[2-ethyl-6-methyl-phenyl]-acetamide, an inhibitor of the biosynthesis of very-long-chain fatty acids (Qin et al., 2007).

In this work, we found that the expression level of GhPIPLC2D was significantly upregulated in the cotton fiber elongation stage and IP3 accumulation was much higher in WT fibers compared to that in WT and fl ovules at 10 days post-anthesis (DPA). Furthermore, silencing GhPIPLC2D reduced fiber length, IP3 accumulation and ethylene content. Exogenous application of linolenic acid and PI, the precursor of PIP2, improved IP3 and IP6 contents as well as ethylene biosynthesis. Exogenous application of IP3, the phosphorylation product of IP6, also significantly enhanced ethylene biosynthesis. These results indicate that
GhPIPLC2D promotes cotton fiber elongation by increasing IP$_3$ accumulation, which in turn stimulates ethylene biosynthesis.

RESULTS

Conserved domains and phylogenetic analysis of GhPIPLCs

In plants, PIPLCs are structurally composed of four conserved domains, the EF-hand-like, PI-PLC-X, PI-PLC-Y, and C2 domains (Abd-El-Haliem and Joosten, 2017). Amino acid sequences of 12 GhPIPLCs were obtained from a previous study (Zhang et al., 2018a). Here, we renamed the GhPIPLCs according to the phylogenetic relationships of GhPIPLCs and AtPIPLCs (Figure 1A); the names and corresponding genome IDs of GhPIPLCs are shown in Table S1. To investigate the sequence conservation of GhPIPLCs, all GhPIPLC members were submitted for analysis by the Pfam online tools (http://pfam.xfam.org/) to obtain more detailed conserved domain information. All GhPIPLCs possessed four domains (Figure 1B) with the exceptions of GhPIPLC1A, GhPIPLC1D, and GhPIPLC6D, which lacked the EF-hand-like domain (Figure S1), indicating that these three GhPIPLCs may be functionally more diverse than the GhPIPLCs that contain all four domains.

In order to explore the evolutionary relationships of PIPLCs, the protein sequences of PIPLCs from G. hirsutum, A. thaliana, G. arboresum, G. raimondii, G. herbaceum, and Oryza sativa were obtained to generate a rooted phylogenetic tree. As shown in Figure 1, GhPIPLC1A and GhPIPLC1D had the longest
evolutionary distances compared with the distances of other GhPIPLCs. There were six GhPIPLCs (GhPIPLC4A-3, GhPIPLC4D-3, GhPIPLC4A-2, GhPIPLC4D-2, GhPIPLC4A-1, and GhPIPLC4D-1), four GaPIPLCs (GaPIPLC4-1, GaPIPLC4-2, GaPIPLC4-3, GaPIPLC4-4), three GhePIPLCs (GhePIPLC4-1, GhePIPLC4-2, GhePIPLC4-3), three GrPIPLCs (GrPIPLC4-1, GrPIPLC4-2, GrPIPLC4-3) and one AtPIPLC4 in the same branch, indicating that GhPIPLC4 may extensively expand in *Gossypium*. To explore the potential driving force of PIPLC4 expansion, we analyzed duplication events in the PIPLC genes and found that tandem duplication is the main contributor to the expansion of PIPLC4 genes in *Gossypium* (Table S2).

**GhPIPLC and IP$_3$ are associated with cotton fiber elongation**

To investigate the potential functions of GhPIPLC genes in cotton, the expression profiles of individual members of GhPIPLCs in cotton fiber and ovules were obtained from CottonFDG (https://cottonfgd.org/) and examined over developmental time from 5 to 25 DPA. The results showed that six members of GhPIPLCs (GhPIPLC4A-2, GhPIPLC4D-2, GhPIPLC4A-3, GhPIPLC4D-3, GhPIPLC2A, and GhPIPLC2D) were predominantly expressed during cotton fiber development. Notably, GhPIPLC2A and GhPIPLC2D had the highest and similar expression patterns in the fiber elongation stage (Figure S2), suggesting these two genes might have similar contributions to cotton fiber development. GhPIPLC2A and GhPIPLC2D were likely homoeologous genes with 84.94% similarity in coding sequences. Therefore, we amplified GhPIPLC2D and checked the sequence specificity via clone sequencing for subsequent functional analyses. The results showed that the GhPIPLC2D coding sequence, but not the GhPIPLC2A coding sequence, was successfully amplified. The expression levels of GhPIPLC2D in different cotton fiber development stages were further confirmed using quantitative real-time polymerase chain reaction (qRT-PCR). As shown in Figure 2A, the expression level of GhPIPLC2D was significantly higher in the fiber elongation stage with peak levels occurring at 5 and 10 DPA than at prior sampling times (Figure 2A), implying the GhPIPLC2D gene may play a critical role in cotton fiber elongation. Furthermore, we detected content of IP$_3$, one of the catalytic products of PIPLC, in fibers and ovules 10 DPA from Xuzhou-142 WT and mutant fl plants and found that IP$_3$ content was higher in 10 DPA WT fibers than that in 10 DPA WT and fl ovules (Figure 2B). Taken together, these results suggest that GhPIPLC2D may promote cotton fiber cell development by regulating IP$_3$ accumulation.

**Silencing GhPIPLC2D in cotton inhibits fiber elongation**

To better understand the biological function of GhPIPLC2D in cotton fiber development, GhPIPLC2D was silenced in *G. hirsutum* using the virus-induced gene silencing (VIGS) strategy. Our results show that the expression level of GhPIPLC2D was clearly reduced in GhPIPLC2D-silenced cotton plants in contrast to the control plants (Figure 3A). We also analyzed the expression of GhPIPLC2A gene in GhPIPLC2D-silenced plants and the results show that GhPIPLC2A transcripts in GhPIPLC2D-silenced plants were similar to the control plants, indicating that GhPIPLC2A transcripts are not decreased in GhPIPLC2D-silenced plants.
We further measured the lengths of mature fibers in GhPIPLC2D-silenced and control plants. GhPIPLC2D-silenced plants displayed shorter fiber length than that in control plants (Figures 3B and 3C). In addition, suppression of GhPIPLC2D expression significantly reduced IP3 accumulation in 10 DPA fiber cells (Figure 3D). These observations are additional evidence of GhPIPLC2D possibly regulating IP3 accumulation, which is essential for cotton fiber cell development.

**GhPIPLC2D gene promotes cotton fiber growth by regulating ethylene biosynthesis**

A previous study demonstrated that ethylene plays a key role in promoting cotton fiber elongation and the 1-aminocyclopropane-1-carboxylic acid oxidase1 (ACO1) and ACO3 genes, two key genes for ethylene biosynthesis, were highly expressed during the fiber growth stage (Shi et al., 2003, 2006). In order to explore the molecular mechanism of GhPIPLC2D in regulating fiber growth, we detected the expression of GhPIPLC2D in GhPIPLC2D-silenced and non-silenced plants. Our results show that the expression of GhACO1 and GhACO3 were significantly down-regulated in GhPIPLC2D-silenced cotton when compared with non-silenced cotton (Figures 4A and 4B).

We also detected ethylene production in GhPIPLC2D-silenced and non-silenced plants. As expected, the accumulation of ethylene was significantly lower in GhPIPLC2D-silenced cotton (Figure 4C). Ethylene accumulation in GhPIPLC2D-silenced plants was reduced to half of that in the non-silenced plants after six days in culture (Figure 4D). These results suggest that the GhPIPLC2D gene may promote cotton fiber growth by regulating ethylene biosynthesis.
development by stimulating the expression of ethylene biosynthesis-related genes and ultimately enhance ethylene production.

Linolenic acid and PI increase IP₃ and IP₆ contents and ethylene biosynthesis

The synthetic precursor of PIP₂ and the catalytic substrate of PIPLC is PI, which is composed of phosphoric acid 1,2-DAG and inositol (Mueller-Roeber and Pical, 2002). Linolenic acid (C18:3) and palmitic acid (C16:0) were the most abundant fatty acids (FA) in PI from the 10 DPA fiber samples. The structural formula of PI biosynthesis is shown in Figure S4.

Carbenoxolone and 5-hydroxytryptamine inhibit the biosynthesis of C18:3 and PI, respectively (Liu et al., 2015). To better understand the effects of C18:3 and PI on cotton fiber cell growth, we detected the amounts of IP₃, IP₆, ethylene and expression of ethylene biosynthesis-related genes after exogenous applications of C18:3, PI, carbenoxolone and 5-hydroxytryptamine to 1 DPA cotton ovules for six days. The results revealed that exogenous application of each C18:3 and PI markedly improved IP₃ accumulation, whereas in vitro application of the corresponding inhibitors, carbenoxolone and 5-hydroxytryptamine, apparently reduced IP₃ accumulation (Figure 5A). The qRT-PCR experiment showed that ethylene biosynthesis-related genes GhACO1 and GhACO3 were significantly upregulated after C18:3 or PI application (Figure 5B). Furthermore, in vitro applications of C18:3 or PI significantly promoted ethylene accumulation,
whereas their corresponding inhibitors dramatically inhibited ethylene production (Figure 5C). After C18:3 or PI treatment for six days, ethylene accumulation nearly increased four times that of the control group. However, the corresponding inhibitor-treated samples decreased ethylene production to half of that of the control (Figure 5D). Meanwhile, although IP6 have higher content in ovules, the IP6 content was increased from 0 DPA and reached a peak at 20 DPA during fiber development (Figure S5), and IP6 accumulation was significantly improved after C18:3 and PI treatments (Figure 5E). These results imply that C18:3 and PI promote IP3 and IP6 accumulation as well as ethylene biosynthesis.

The C18 fatty acid contains saturated fatty acid C18:0 and unsaturated fatty acids C18:1, C18:2, and C18:3 (Conte et al., 2018). To investigate whether other C18 fatty acids could stimulate IP3 accumulation, the levels of IP3 in ovules treated with C18:0, C18:1, C18:2, or C18:3 were measured. Exogenous application of C18:0, C18:1, and C18:2 did not increase IP3 contents compared with that of the control; only in vitro applications of C18:3 and PI promoted IP3 accumulation as well as ethylene biosynthesis.

**Figure 5.** C18:3 and PI promote IP3 and IP6 production and ethylene biosynthesis
Accumulation of IP3 (A) and ethylene biosynthesis gene transcripts (B) in ovules treated in vitro with C18:3, the C18:3 inhibitor carbenoxolone, PI or the PI inhibitor 5-hydroxytryptamine. Gene transcripts in (B) were obtained by qRT-PCR with three replicates.
(C) ethylene production in the same treatments as in (A).
(D) ethylene production over six days of ovule cultivation with the same treatments as in (A).
(E) IP6 accumulation in ovules treated in vitro with C18:3, the C18:3 inhibitor carbenoxolone, PI or the PI inhibitor 5-hydroxytryptamine. *p < 0.05, **p < 0.01, ***p < 0.001. Statistical significance was determined using one-way ANOVA with Tukey’s test. Error bars represent the SE (n = 3 biological replicates). No chemicals were added the control.
application of C18:3 improved IP3 accumulation (Figure S6). We further analyzed the total fatty acid signal intensities extracted from different tissues of cotton. The results showed that C16:0, C18:2, and C18:3 were the most abundant fatty acids in flowers, leaves, and ovules. Moreover, flowers, leaves, and ovules also contained higher amounts of total fatty acids than that from roots and stems (Figure S7).

IP6 improves fiber length and ethylene biosynthesis
Catalysis of PIP2 by PIPLC produces IP3, which can be further phosphorylated to form IP6 (Gibson et al., 2018). In order to determine whether IP6 potentially regulates cotton fiber elongation and ethylene biosynthesis, we measured cotton fiber length, ethylene biosynthesis-related gene expression and the amount of ethylene accumulation in response to different concentrations of IP6 treatment. We observed an increase of fiber length in a dose-dependent manner with the increase of IP6 concentrations from 1 to 10 μM (Figure 6A). Fiber length increased three-fold that of the control group after treatment with 10 μM IP6. Furthermore, exogenous application of IP6 increased the expression of GhACO1 (Figure 6B) and GhACO3 (Figure 6C). As expected, ethylene accumulation (Figure 6D) also increased after IP6 treatment in vitro. These results suggest that IP6 can promote fiber elongation and ethylene biosynthesis.

Ethylene and IP6 significantly promoted fiber cell elongation in GhPIPLC2D-silenced cotton
To further understand the biological role of IP6 and ethylene on cotton fiber cell development, WT and GhPIPLC2D-silenced cotton ovules collected at 1 DPA were cultured with 5 μM IP6 and 0.01 μM ethylene for 6 days. Subsequently, the length of fiber cells was observed and measured in microscope. The result showed that exogenous application of ethylene and IP6 significantly enhanced the fiber length of GhPIPLC2D-silenced cotton and WT (Figure 7A). Furthermore, the fiber length of GhPIPLC2D-silenced plants treated with ethylene and IP6 was obviously longer than the samples without any treatment (Figure 7B). These results suggest that ethylene and IP6 can recover fiber length shortened by GhPIPLC2D gene silencing.
DISCUSSION

The PIPLC gene family contains nine members in Arabidopsis (Tasma et al., 2008), four members in rice (Singh et al., 2013) and twelve members in G. hirsutum (Zhang et al., 2018a). The PIPLC protein is usually composed of four conserved domains (Abd-El-Haliem and Joosten, 2017). In G. hirsutum, GhPIPLCs contained four conserved domains, except for GhPIPLC1A, GhPIPLC1D and GhPIPLC6D, which lacked the EF-hand-like domain (Figure S1). Interestingly, mutating an EF-hand-like domain of PIPLC did not affect Ca²⁺-dependent substrate hydrolysis in Dictyostelium discoideum (Drayer et al., 1995), suggesting the domain may not be a regulatory site of the Ca²⁺ dependence of the PIPLC reaction, although the EF-hand-like domain is required for enzyme activity.
Phylogenetic analysis showed that six GhPIPLC4s, four GaPIPLC4s, three GhePIPLC4s, three GrPIPLC4s and one AtPIPLC4 were in the same evolutionary branch (Figure 1), indicating that the PIPLC4 sequence might have expanded in Gossypium. In Arabidopsis, the expression of AtPIPLC4 is positively upregulated after salt stimulation (Tasma et al., 2008). OsPIPLC1 prefers to hydrolyze PIP2 and elicits stress-induced Ca^{2+} signals to regulate salt tolerance (Li et al., 2017). Meanwhile, cotton is a moderately salt-tolerant crop with a salinity threshold level of 7.7 dS m^{-1} and has a higher basal level of tolerance to NaCl compared to that of other major crops (Sharif et al., 2019, Li et al., 2015). The moderate level of salt tolerance implies that GhPIPLC4A-1, GhPIPLC4D-1, GhPIPLC4A-2, GhPIPLC4D-2, GhPIPLC4A-3, and GhPIPLC4D-3 may have an important role in salt stress response in cotton development, and the salt stress may be the driving force in the expansion of these six genes during the evolutionary process. To verify the role of these six GhPIPLCs in salt stress response, further investigations in the future, such as genetic verification experiments, are needed.

PI-specific phospholipase C is the key enzyme that catalyzes PIP2 to produce IP3 and DAG (Kadamur and Ross, 2013). IP3, the critical secondary messenger that mediates calcium release from the ER, serves as the precursor in inositol phosphate biosynthesis and can be phosphorylated to form IP6. Thus, IP3 affects the downstream regulatory pathway of phytic acid (Xia and Yang, 2005). In this study, we discovered that Ip3 content in WT fibers was higher than that in WT and fl ovaules at 10 DPA (Figure 2). Silencing GhPIPLC2D gene expression reduced Ip3 content and fiber length (Figure 3). These results suggest that Ip3 may contribute to cotton fiber elongation, which could be confirmed by observing the phenotypes resulting from stably transformed cotton plants. In addition, the GhPIPLC2D and GhPIPLC2A are allele and had similar expression patterns (Figure S2), suggesting both two genes might have similar functions. Silencing both GhPIPLC2D and GhPIPLC2A genes might have fiber length shorter than silencing only GhPIPLC2D, which needs to be further investigated.

A previous study revealed that linolenic acid promotes fiber elongation by activating PI and PIP biosynthesis (Liu et al., 2015). In eukaryotic cells, PI is the major phospholipid involved in a wide range of signaling pathways, such as hormone regulation, biotic and abiotic stress responses, and light response. PI is mainly phosphorylated to PIP2, and then PIP2 is cleaved to form IP3 and DAG, which are two important secondary messengers in cells (Abd-El-Haliem and Joosten, 2017). Our data showed that exogenous application of C18:3 and PI significantly increased Ip3 and Ip6 contents, while in vitro applications of their inhibitors expectedly reduced Ip3 and Ip6 accumulation (Figure 5). Exogenous application of Ip6 significantly promoted cotton fiber length and the expression of ethylene biosynthesis genes (Figure 6). These results further indicate that Ip3 and Ip6 might play a critical role in cotton fiber elongation. The Ip6 content measurement also showed that the Ip6 content was increased during fiber development (Figure S5). Meanwhile, exogenous applications of ethylene and Ip6 significantly improve the fiber length in GhPIPLC2D-silenced plant (Figure 7). Our study revealed that the GhPIPLC2D gene acts as a positive regulator in cotton fiber elongation, which the enzyme it encodes catalyzes PIP2 to DAG and IP3. Furthermore, IP3 is phosphorylated to form IP6 to promote cotton fiber elongation (Figure 8). In addition, previous study showed that phytic acid is mainly accumulated in the embryo of seed in maize, and it mainly provide phosphate and minerals for use during seedling growth and germination (Shi et al., 2003). The phosphorus was translocated to seed from roots and leaves and for synthesizing phytic acid and stored in seeds and it breakdown during germination for early seedling growth (Taliman et al., 2019). The 10 DPA ovaules have the highest Ip6 content (Figure S5), indicating that Ip6 may also play important roles in ovaule development.

Ethylene is a major phytohormone that participates in many developmental stages, such as cell division and root hair development (Song et al., 2019). In cotton, ethylene plays a major role in fiber cell elongation (Shi et al., 2003, 2006). One study showed that very-long-chain fatty acids promote fiber elongation by enhancing ethylene biosynthesis (Qin et al., 2007). In this study, we found that the expression of GhACO1 and GhACO3, as well as ethylene production, were significantly decreased in GhPIPLC2D-silenced cotton compared with those of WT cotton (Figure 4). In addition, exogenous application of linolenic acid (C18:3), PI and Ip6 promoted ethylene biosynthesis (Figure 5). These results indicate that GhPIPLC2D and Ip6 promoted cotton fiber cell development possibly by activating ethylene biosynthesis and enhancing ethylene accumulation (Figure 8). This study provides empirical evidence that Ip3 regulates ethylene biosynthesis and promotes cotton fiber development, which is a branch in ethylene regulation of cotton fiber growth.

Calcium signals have been found to contribute to cotton fiber development (Guo et al., 2017). In Arabidopsis, PIPLC has been shown to be important in Ca^{2+} signaling, and piplc3 mutants showed decreased Ca^{2+}
Release (Gao et al., 2014). The reductions of IP3 and IP6 levels affect Ca2+ release from the cytosol and might contribute to flg22-dependent cytosolic Ca2+ bursts (Hilleary et al., 2020). Moreover, Hasenstein and Evans (1986) found that Ca2+ enhances the conversion of 1-aminocyclopropane carboxylic acid (ACC) to ethylene in primary roots of corn. Yu et al. (2019) showed that Ca2+ promotes root development in response to salt stress by regulating the biosynthesis of ethylene. As a secondary messenger, Ca2+ is central for plant signal transduction. Calcium is involved in most environmental responses and phytohormone signal pathways (Peiter, 2011; Guo et al., 2017). Therefore, we speculate that GhPIPLC2D may also affect Ca2+ release and thus participate in fiber development in cotton. In the future, molecular mechanisms and regulatory relationships between GhPIPLC2D-Ca2+-ethylene in regulating fiber cell elongation should be examined to deepen our understanding of the underlying processes in cotton fiber development.

Limitations of the study
In this study, we revealed a GhPIPLC2D gene serves as a positive regulator in cotton fiber elongation, which catalyzes PIP2 to produce IP3 and IP3 promotes fiber elongation by enhancing ethylene biosynthesis. However, as we have discussed in the article, the GhPIPLC2D expression impact IP3 content and IP3 accumulation promote fiber elongation through enhancing ethylene biosynthesis while the GhPIPLC2D-ethylene in regulating fiber cell elongation should be examined to deepen our understanding of the underlying processes in cotton fiber development. In addition, how IP3 promotes ethylene synthesis also needs to be further clarified in future studies.

Resource availability
Lead contact
Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Guanghui Xiao (guanghuix@snnu.edu.cn).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate or analyze data sets and code.

METHODS
All methods can be found in the accompanying Transparent methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102199.
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AUTHOR CONTRIBUTIONS

L.Z. and D.L. performed the experiments; L.Z. and H.S. analyzed the data; L.Z., D.L., and H.S. performed software application and data visualization; L.Z. and G.X. wrote the paper; G.X., J.Y., and H.L. conceived and designed the experiments. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare that they have no conflict of interests.

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Supplemental information

*GhPIPLC2D* promotes cotton fiber

elongation by enhancing

ethylene biosynthesis

Liping Zhu, Lingling Dou, Haihong Shang, Hongbin Li, Jianing Yu, and Guanghui Xiao
Supplemental Information

Transparent Methods

Phylogenetic and domain analysis of GhPIPLCs

Multiple amino acid sequences of PIPLCs from *A. thaliana* (Tasma et al., 2008), *O. sativa* (Singh et al., 2013), *G. arboreum* (Du et al., 2018), *G. herbaceum* (Huang et al., 2020), *G. raimondii* (Wang et al., 2012) and *G. hirsutum* (Zhang et al., 2018a) were obtained to construct a phylogenetic tree using MEGA 7.0 software (Kumar et al., 2016) and the neighbor-joining statistical method with 1000 bootstrap replications. The GhPIPLCs were renamed according to the phylogenetic relationships of GhPIPLCs and AtPIPLCs; the names and corresponding genome ID information of GhPIPLCs are shown in Supplementary Table 1. The amino acid sequences of all GhPIPLCs were then submitted to an online bioinformatic tool Pfam (http://pfam.xfam.org/) to investigate the conserved domain information.

Plant materials and *in vitro* ovule culture

The cultivar of upland cotton Xuzhou-142 wild-type (WT) and its fuzzless-lintless mutant (*fl*) produced from WT plants as well as GhPIPLC2D-silenced plants were grown in a greenhouse at 60% humidity, 25 °C, and a 16-h/8-h light/dark cycle. Cotton bolls were picked at -3, 0, +3, +5, +10, +15 and +20 days post-anthesis from both WT and mutant plants. The fibers and ovules at -3 to +20 DPA were stored in liquid nitrogen until use. The XJ128 Rapid Fiber Tester (ChangLing, China) was used for fiber length detection following the standard test methods of the manufacturer. Ovules were obtained at anthesis and then sterilized using 10% sodium hypochlorite solution prior to culturing. For the C18 fatty acid, PI, IP6 and ethylene treatment assays, 5 µM of each C18:0, C18:1, C18:2, C18:3, and PI; 0.5–1 µM of the C18:3 inhibitor carbenoxolone; 0.5–1 µM of the PI inhibitor 5-hydroxytryptamine; or 1–10 µM of IP6 and 2 µM of ethephon were cultured with cotton ovules in the culture medium formulated by Beasley and Ting (1973) at 30 °C under aseptic conditions (Shi et al., 2006). The composition of the culture medium was as follows: 272.18 mg/L KH2PO4, 6.183 mg/L H3BO3, 0.242 mg/L Na2MoO4·2H2O, 441.06 mg/L CaCl2·2H2O, 0.83 mg/L KI, 0.024 mg/L CoCl2·6H2O, 493 mg/L MgSO4·7H2O, 16.902 mg/L MnSO4·H2O, 8.627 mg/L ZnSO4·7H2O, 0.025 mg/L CuSO4·5H2O, 5055.5 mg/L KNO3, 8.341 mg/L FeSO4·7H2O, 11.167 mg/L Na2EDTA, 0.492 mg/L nicotinic acid (vitamin B3), 0.822 mg/L pyridoxine·HCL (vitamin B6), 1.349 mg/L thiamine·HCL (vitamin B1), 180.16 mg/L myo-inositol, 18016 mg/L D-glucose and 3603.2 mg/L D-fructose. The pH was 6.0.
**RNA extraction and quantitative real-time PCR**

Total RNA from fibers and ovules were extracted using the Invitrogen RNeasy kit (Life Technologies, USA). First-strand complementary DNA (cDNA) were reverse-transcribed from 2 µg total RNA using the standard procedure described in the kit’s manual, including the DNase treatment steps (Takara, Japan). The qRT-PCR was performed using gene-specific primers listed in Supplementary Table 3. We used *UBQ7* (GenBank No. AY189972) as the internal control. The reactions, with samples having three technical replicates, were performed using the Roche Light Cycle 480 II instrument (Roche, Basel, Switzerland). One reaction contained 0.5 µL cDNA (10 ng), 10 µL SYBR/ROX qPCR Mix (2×), 0.75 µL forward primer, 0.75 µL reverse primer and 8 µL ddH2O. The qPCR reaction was performed as follows: 95°C for 3 min followed by 40 cycles of 95°C for 25 s, 56°C for 30 s and 72°C for 30 s. Fluorescence signals were automatically acquired at the end of each cycle. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression levels of the target genes. Three independent biological replications were carried out for each gene. The Multi Experiment Viewer (MeV, version 4.9, Boston, MA, USA) software was used to generate gene expression heat maps.

**Virus-induced gene silencing (VIGS) and cotton plant transformation**

We used *Cotton leaf crumple virus* (CLCrV)-based vectors (i.e., pCLCrV-A and pCLCrV-B) for the VIGS experiment (Gu et al., 2014). The 429-bp *GhPIPLC2D* gene fragment was amplified from total cDNAs with primers listed in Supplementary Table S3. A total of nine plants were used for the VIGS experiment, consisting of three biological replicates. The PCR products were digested with SpeI and Ascl (detailed information of the restriction sites are attached in Supplementary Table 3) and then ligated into the pCLCrV-A vector using NEB T4 DNA ligase (New England BioLabs, Ipswich, MA). We followed the manufacturer’s protocol supplied with the ligase. The constructs (pCLCrV: *GhPIPLC2D*, pCLCrV-A, and pCLCrV-B) were individually introduced into *Agrobacterium tumefaciens* strain LBA4404. The *Agrobacterium* colonies containing pCLCrV: *GhPIPLC2D*, pCLCrV-A, or pCLCrV-B were grown for 24 h at 28 °C. Then the *Agrobacterium* cells were collected and resuspended in infiltration medium (10 mM MgCl2, 10 mM MES, and 200 mM acetosyringone) and cultured to OD600 = 1.2. The *Agrobacterium* cells containing pCLCrV-B were mixed with either the culture of *Agrobacterium* cells with pCLCrV: *GhPIPLC2D* or pCLCrV-A at a ratio of 1:1. Each mixture was injected into three cotton seedling cotyledons (about 10-day-old seedlings) for the silencing experiment. After a 24-h incubation in darkness, the seedlings were grown at 25°C under a 16-h light, 8-h dark cycle.
IP₃ content determination

Extraction of IP₃ was performed using a method described previously (Burnette et al., 2003). The IP₃ content was determined by using the Inositol-1,4,5-Trisphosphate [³H] Radioreceptor Assay Kit (PerkinElmer Life Sciences, Finland) and a standard curve derived from known concentrations of IP₃.

Cotton fatty acid extraction and Gas chromatography–mass spectrometry

Fatty acids were extracted from 20 mg flower, leaf, root and stem tissues from four-month-old cotton plants and from the ovules at 10 days post-anthesis. Subsequently, they were freeze-dried and then immersed in chloroform/methanol (2:1, v/v) for 1 min to remove surface waxes (Qin et al., 2007). Cotton samples were homogenized in liquid nitrogen and extracted with 2.5% H₂SO₄ in methanol (v/v). Heptadecanoic acid (C17:0), as the internal standard, was added into the fatty acid extraction medium to monitor fatty acid recovery and quantification. Then the fatty acid methyl esters were dissolved in hexane. A 1 µL sample was injected into the Agilent 6890N GC system (Agilent, California, USA). Fatty acids were measured by an HP 5975 mass selective detector (HP, California, USA) connected to the GC system using the method described previously (Liu et al., 2015).

Ethylene content measurements

Twenty 1 DPA ovules were freshly-collected and then cultured in a 96-well culture plate with 150 µL liquid media containing 5 µM linolenic acid, 2 µM PI, 1 µM carbenoxolone or 1 µM 5-hydroxytryptamine for 6 d at 30°C. Air samples (100 µL) from each well were collected with a sample injector and injected into the gas chromatograph column held at 60°C for 20 min with nitrogen as the carrier gas. A gas chromatograph (GC6890N; Agilent) equipped with a flame-ionization detector and a HP-PLOT column (30 m × 530 µm × 40 µm, Agilent Technologies) was used to perform ethylene measurements. Standards of 0.1, 1, 10, and 50 ppm ethylene were used to determine the amount of ethylene production. All experiments were performed with three replicates. Statistical significance was determined using one-way ANOVA with Tukey’s test in this research.

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Figure S1. Phylogenetic and conserved domain analysis of GhPIPLCs, Related to Figure 1.

Gray lines indicate protein sequence lengths. Boxes with different colors represent different conserved domains. Numbers under the boxes show the specific location of each domain.
Figure S2. Expression profiles of *GhPIPLC* over time of fiber and ovule development, Related to Figure 2.

Expression patterns of genes were clustered. Green and red colors indicate low and high transcriptional expression levels, respectively. The *GhPIPLC2D* genes (in red font) were selected for further functional analysis. DPA, days post-anthesis.
Figure S3. Relative expression levels of *GhPIPLC2A* in *GhPIPLC2D*-silenced cotton, Related to Figure 3.

Gene expression data were obtained by quantitative real-time PCR with three independent replicates. Statistical significance was determined using one-way ANOVA with Tukey's test. Error bars represent the SE (n = 3 biological replicates).
Figure S4. Structural formula of phosphatidylinositol (PI) biosynthesis, Related to Figure 5.

C16:0, palmitic acid. C18:3, linolenic acid. PIS, phosphatidylinositol synthase. CDP-DAG, CDP-diacylglycerol. DPA, days post-anthesis. The two fatty acids in the red box represent the most abundant fatty acids of PI in 10 DPA fiber.
Figure S5. Accumulation of IP₆ in ovules and fibers at different developmental stages, Related to Figure 5.

Statistical significance was determined using one-way ANOVA with Tukey's test. Error bars represent the SE (n = 3 biological replicates). **P < 0.01. No chemicals were added to the control.
Figure S6. Accumulation of IP$_3$ after exogenous application of C18:0, C18:1, C18:2 or C18:3, Related to Figure 5.

Statistical significance was determined using one-way ANOVA with Tukey's test. Error bars represent the SE (n = 3 biological replicates). **$P < 0.01$. No chemicals were added to the control.
Figure S7. Fatty acid accumulation in five different cotton plant tissues, Related to Figure 5.

(A) intensities of different types of fatty acids.
(B) intensities of total fatty acids. Error bars represent the SE (n = 3 biological replicates).
Table S1. Given names (New Name) of *GhPIPLCs* used in this study corresponding to their names and genome IDs obtained from Zhang et al. (2018a), Related to Figure 1.

| New Name | Name       | ID          |
|----------|------------|-------------|
| GhPIPLC1D | GhPIPLC4   | CotAD_09433 |
| GhPIPLC1A | GhPIPLC9   | CotAD_18525 |
| GhPIPLC2A | GhPIPLC5   | CotAD_62184 |
| GhPIPLC2D | GhPIPLC11  | CotAD_22832 |
| GhPIPLC4A-1 | GhPIPLC3 | CotAD_09434 |
| GhPIPLC4D-1 | GhPIPLC8 | CotAD_18524 |
| GhPIPLC4A-2 | GhPIPLC2 | CotAD_09425 |
| GhPIPLC4D-2 | GhPIPLC7 | CotAD_18522 |
| GhPIPLC4A-3 | GhPIPLC1 | CotAD_56315 |
| GhPIPLC4D-3 | GhPIPLC6 | CotAD_30245 |
| GhPIPLC6D | GhPIPLC10  | CotAD_22531 |
| GhPIPLC7D | GhPIPLC12  | CotAD_22314 |
Table S2. Analysis of duplication events of PIPLC4 genes from *G. hirsutum*, *G. arboreum*, *G. herbaceum* and *G. raimondii*, Related to Figure 1.

| Accession     | Gene          | Type                  |
|---------------|---------------|-----------------------|
| CotAD_56315   | GhPIPLC4A-3   | Whole Genome/Segmental Duplication |
| CotAD_30245   | GhPIPLC4D-3   | Whole Genome/Segmental Duplication |
| CotAD_09425   | GhPIPLC4A-2   | Tandem duplication     |
| CotAD_09434   | GhPIPLC4A-1   | Tandem duplication     |
| CotAD_18522   | GhPIPLC4D-2   | Tandem duplication     |
| CotAD_18524   | GhPIPLC4D-1   | Tandem duplication     |
| Cotton A 12942| GaPIPLC4-1    | Tandem duplication     |
| Cotton A 12940| GaPIPLC4-4    | Tandem duplication     |
| Cotton A 21123| GaPIPLC4-2    | Tandem duplication     |
| Cotton A 21120| GaPIPLC4-3    | Tandem duplication     |
| Ghe05G09560   | GhePIPLC4-1   | Tandem duplication     |
| Ghe05G09580   | GhePIPLC4-2   | Tandem duplication     |
| Ghe09G13250   | GhePIPLC4-3   | Dispersed             |
| Gorai.009G091700.1| GrPIPLC4-1 | Tandem duplication     |
| Gorai.009G091900.1| GrPIPLC4-2 | Tandem duplication     |
| Gorai.006G106400.1| GrPIPLC4-3 | Dispersed             |
### Table S3. Primers used in this work, Related to Figure 2, Figure 3, Figure 4, Figure 5, Figure 6.

| Name          | Sequences                                                                 |
|---------------|---------------------------------------------------------------------------|
| **VIGS of GhPIPLC2D (endonuclease)** |                                                                            |
| **GhPIPLC2D** | 5'-GGACTAGTTTGGAGACATCCTGTTTTACCT (Spe I)                                  |
|               | 5'-TGGCGCGCCCTTCCCTGAGTAATCGACGATCAT (Ascl)                                |
| **qRT-PCR analysis** |                                                                 |
| **GhACO1**   | 5'-TAATCACAATGGTAAATATA                                                   |
|               | 5'-TCGAACCTTGGCTCCTTGGC                                                   |
| **GhACO2**   | 5'-CAATCCTGGAAGTGATGCTGTT                                                 |
|               | 5'-CGAACCTCGCTCCTTGCT                                                    |
| **GhACO3**   | 5'-AAGAGTGAGGAGCAGCCAGTC                                                 |
|               | 5'-TTTCTTTCACCCAACCAGCC                                                   |
| **GhACO4**   | 5'-GCCATCTCCCTGAATCAAACA                                                |
|               | 5'-TTTATCTGGGTGGGCCC                                                     |
| **GhACS2**   | 5'-AAAGCCTACGACAGCCAGCCTT                                                |
|               | 5'-CATAACTATACGGTTCCCATCA                                                  |
| **GhACS3**   | 5'-ATGGGGAAAGTGAGGGGAGA                                                 |
|               | 5'-TGCCAACTCTAAAAACAGGGGAAC                                                |
| **GhACS4**   | 5'-GTGCCCGCAAATGTCCA                                                     |
|               | 5'-GGAAAGAAAGAAGCTCGGCCAACC                                                |
| **GhACS6**   | 5'-AAGTCGATCGTTGGAAGGAGGC                                               |
|               | 5'-GGTGAAGTTGAGGTAGGGAGAGTGG                                            |
| **GhACS10**  | 5'-GTATGACAGGGATGAAAAATGGA                                               |
|               | 5'-TTTCTTCTCTCTGGCAAAGTCAA                                                  |
| **GhACS12**  | 5'-CGCTTTATCTACTTTCACTCCAATCTCT                                         |
|               | 5'-TTTCAATCTAAATCTCAACACAACC                                               |
| **GhPIPLC2A**| 5'-TTCAAGAGTTCTCCCTGTC                                                  |
|               | 5'-ATCATCTGTATTTCTCT                                                       |
| **GhPIPLC2D**| 5'-CTGAAGGAATTCCCTGCTCT                                            |
|               | 5'-CTTATCTCCATCGTCAATCGAG                                                |
| **GhUBQ7**   | 5'-GAAGGCATTCCAACGACCCAC                                             |
|               | 5'-CTTGACCTTCTTTCTTGCTCT                                                   |