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The crosstalk between Target of Rapamycin (TOR) and Jasmonic Acid (JA) signaling existing in Arabidopsis and cotton

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Target of rapamycin (TOR) acts as an important regulator of cell growth, development and stress responses in most examined diploid eukaryotes. However, little is known about TOR in tetraploid species such as cotton. Here, we show that TORC1-S6K-RPS6, the major signaling components, are conserved and further expanded in cotton genome. Though the cotton seedlings are insensitive to rapamycin, AZD8055, the second-generation inhibitor of TOR, can significantly suppress the growth in cotton. Global transcriptome analysis revealed that genes associated with jasmonic acid (JA) biosynthesis and transduction were significantly altered in AZD8055 treated cotton seedlings, suggesting the potential crosstalk between TOR and JA signaling. Pharmacological and genetic approaches have been employed to get further insights into the molecular mechanism of the crosstalk between TOR and JA. Combination of AZD8055 with methyl jasmonate can synergistically inhibit cotton growth, and additionally JA levels were significantly increased when cotton seedlings were subjected to AZD8055. JA biosynthetic and signaling mutants including jar1, coi1-2 and myc2-2 displayed TOR inhibitor-resistant phenotypes, whereas COI1 overexpression transgenic lines and jaz10 exhibited sensitivity to AZD8055. Consistently, cotton JAZ can partially rescue TOR-suppressed phenotypes in Arabidopsis. These evidences revealed that the crosstalk between TOR and JA pathway operates in cotton and Arabidopsis.

Target of rapamycin (TOR) is a serine/threonine protein kinase and is evolutionally conserved from the last eukaryotic common ancestor (LECA) to humans1–4. TOR proteins in these diverse species consist of several highly conserved signature domains including HEAT (Huntingtin, elongation factor 3, regulatory subunit A of PP2A, TOR1) repeats, FAT (the FRAP, ATM, and TRRAP) domain, FRB (FKBP12-rapamycin-binding) domain, kinase domain and FATC (Carboxy-terminal FAT) domain1,4. In yeasts and mammals, TOR is present in two functionally distinct complexes named TORC1 (TOR complex 1) and TORC2 (TOR complex 2). TORC1 contains TOR, regulatory-associated protein of mTOR (RAPTOR) and lethal with SEC13 protein 8 (LST8), and is rapamycin sensitive. TORC2, which is rapamycin insensitive, includes TOR, LST8, rapamycin-insensitive companion of mTOR (RICTOR) and stress activated map kinase-interacting protein 1 (SIN1)3. Rapamycin (RAP) is an antibiotic produced by Streptomyces hygroscopicus, and has aided a wealth of studies in animals and yeasts1,3. However, TOR is insensitive to RAP in majority of land plants for instance Arabidopsis and Vicia faba, likely due to the altered structural aspects of FKBP12 (FK506 binding protein of 12 kDa) in the formation of functional TOR-rapamycin-FKBP12 ternary complex5,6.

Compared to great progress made in the understanding of TOR signaling in animals and yeasts, relatively less is known about this ancient and important regulatory system in higher plants. To explore TOR functions using RAP in higher plants, yeast FKBP12-based Arabidopsis transgenic plants (BP12-2) were produced, which confer conditional sensitivity to rapamycin7. Recent studies have also revealed that the ATP-competitive TOR specific

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inhibitors, which were named after active-site TOR inhibitors (asTORis) including AZD8055 (AZD), Torin1 and Torin2, can also effectively inhibit TOR activity and retard plant growth\(^8\). These inhibitors are effective against both TORC1 and TORC2 complexes and have broader kinase-dependent inhibition on TOR than the widely used rapamycin. Since TOR has many upstream signaling inputs and downstream signaling outputs in eukaryotic organisms, and different signaling pathways associated with these confer different functions, both rapamycin and asTORis could potentially be applied for advancing the underpinning mechanisms in plants. A recent transcriptome analysis of Arabidopsis seedlings exposed to AZD showed that TOR regulated photosynthesis and phytohormone signaling pathways including jasmonic acid (JA) signaling pathway\(^4\). Although these results implied the potential cross-talk between TOR and JA, direct experimental evidence supporting this interaction remains largely elusive.

The phytohormone JA regulates a broad spectrum of biological processes, including cell growth and development, as well as defense responses to biotic and abiotic stresses\(^12,13\). In the past decades, remarkable progress has been made in understanding of JA biosynthesis and its signaling transduction\(^14,15\). The bioactive jasmonoyl-isoleucine (JA-Ile), whose formation is catalyzed by JAR1 (Jasmonoyl isoleucine conjugate synthase1), is perceived by the SCF\(^\text{COI1}\) (Skp1/Cullin/F-box) complex that contains CORONATINE-SENSITIVE1 (COI1) F-box protein and the transcript repressor JASMONATE-ZIM DOMAIN (JAZ) protein\(^16-22\). After perception, JAZ proteins were degraded through the 26S proteasome. The bHLH (basic helix-loop-helix) transcription factors MYC2 (Myc transcription factor 2) and MYC3 (Myc transcription factor 3) are the most well characterized regulatory components targeted by JAZ\(^9\).

Cotton (Gossypium hirsutum) provides the major resource of textile fiber worldwide. Environmental stresses are major threats to cotton crop yields. To deal with environmental stresses, crop plants like cotton frequently encounter yield penalties resulting from effects on growth and development. Relevant to these, TOR is involved in regulating plant growth and JA plays a key role in response to environmental stresses. To deal with environmental stresses, crop plants like cotton frequently encounter yield penalties resulting from effects on growth and development. Relevant to these, TOR is involved in regulating plant growth and JA plays a key role in response to environmental stresses\(^2-4\). Dissecting the underlying genetic components and the mechanistic insights linking TOR and JA in plants may provide alternate approaches to develop tolerance against environmental stress and thereby reduce production losses. In this study, we addressed this issue and established that TOR signaling had a significant influence on JA biosynthesis and the associated signal transduction pathways in cotton and Arabidopsis. Our results provide new evidences showing a connection between TOR and JA signaling pathways and demonstrate that TOR has a negatively effect on JA signaling pathway.

**Results**

**TOR signaling pathway in G. hirsutum.** The availability of full genome sequences across large number of eukaryotes has significantly expanded our knowledge of TOR signaling pathway\(^2\). Recently sequenced Gossypium hirsutum genome has provided us an opportunity to identify these evolutionarily conserved TOR signaling pathway components in tetraploid species cotton\(^23\). We found the putative homologous gene sequences encoding the key proteins of TORC1 complex including TOR, RAPTOR, and LST8; however, no putative homologs of TORC2-specific proteins, such as RICTOR and SIN1, were present in cotton genome (Table 1 and Supplementary Table 1). Further analysis revealed that the homologs of HsTOR were presented at two genetic loci in G.hirsutum genome (Fig. 1A). The full-length gene sequence of GhTOR1 (Target of Rapamycin 1) spans 20,012 bp and contains 58 exons, and GhTOR2 (Target of Rapamycin 2) has 21,569 bp with the same number of exons as observed in Arabidopsis. Arabidopsis protein sequences showed similar abiotic stress response and yeasts, whereas the FATC domain was not detected in GhTOR2 protein (Fig. 1B). Phylogenetic analysis (Fig. 1C) and kinase domain sequence alignment with that from other representative organisms (Fig. 1D) indicated that GhTOR1 and GhTOR2 were evolutionarily conserved. Moreover, QRT-PCR (Quantitative real time PCR) analysis revealed that the expression of these two GhTOR genes can be detected with similar profiles in all cotton tissues (Fig. 1E). It should be noted that besides the two TOR homologs, four putative RAPTOR homologs were found, but only one copy of LST8 and FKBP12 can be detected in cotton genome (Supplementary Table 1). These observations indicate that TOR and RAPTOR have been duplicated during the evolution of cotton genome from diploid to heterotetraploid whereas one copy of LST8 and FKBP12 was lost during this process, indicating that TOR and RAPTOR may play more crucial parts in the evolutionary history and life strategies of cotton (Supplementary Table 1).

IRS (Insulin receptor substrate)-PISK (Phosphoinositide 3-kinase)-PKD (3’-phosphoinositide-dependent protein kinase)-Akt-TSC1 (Tuberous sclerosis 1)/TSC2 (Tuberous sclerosis 2) signaling cascade is a crucial upstream regulatory input of TOR in humans\(^4,4\). Except PKD, no homologs of other components were present in cotton (Table 1). Since LKB (Loss of liver kinase B1)-AMPK (AMP-activated protein kinase) is another upstream signaling component of TORC1\(^26,27\), we investigated the corresponding putative homologs of LKB and AMPK. Two copies of LKB and three copies of AMPK were identified and they all displayed high similarities with the corresponding Arabidopsis homologs (Supplementary Table 1), indicating that LKB-AMPK, the major energy sensor for ATP/AMP, is a shared signaling factor upstream of TOR in Arabidopsis and cotton. Furthermore, our analysis also identified the downstream targets of TOR that include the homologs of S6K (S6 kinase), RPS6 (Ribosomal protein S6), c4, E2F3 (E2F transcription factor 3), ATPB3D (Repressor of LRR-extension 1), and TCTP (Translational controlled tumor protein); however, the homologs of VPS34 (Vacuolar protein sorting 34), 4EBP1 (Translation initiation factor 4E-binding protein 1), and SGK1 (Shuttle mutant glucocorticoid-regulated kinase) were not detected (Table 1 and Supplementary Table 1). It should be noted that additional copies of S6K (6 copies) and RPS6 (ten copies) have been found, suggesting the expansion of these genes in cotton genome compared to the other TOR components like c4 (1 copy), E2F3 (2 copies), ATPBD3 (1 copy) and TCTP (2 copies) in cotton (Supplementary Table 1). The extensive expansion of S6K-like and RPS6-like genes along with the duplications of TOR and RAPTOR suggests that TOR/RAPTOR-S6K-RPS6 signaling cascade may act as the mainstay of highly conserved TOR signaling pathways in controlling cell growth and proliferation in cotton.
AZD but not RAP can efficiently inhibit the growth of cotton seedlings in a dose dependent manner. RAP is a well-known TOR inhibitor and is extensively employed in deciphering plant TOR functions.8,9 We analyzed the sequences of GhFKBP12 in the cotton genome (http://cgp.genomics.org.cn). Only a single copy of FKBP12 (CotAD_57498) was present and was evolutionary conserved among species (Supplemental Fig. 1A). Besides, we performed real-time PCR analyses of GhFKBP12 and found that GhFKBP12 had a similar expression pattern to that of GhTOR (Supplemental Fig. 1B). Next, to get functional insights into this gene, cotton seedlings and GhFKBP12 overexpression transgenic Arabidopsis lines were exposed to RAP treatment (Supplemental Fig. 1C–H). They all displayed insensitivity to rapamycin, and this observation was consistent with that reported in Arabidopsis.6,28 Sequence analysis of GhFKBP12 and homologs of other organisms indicated that alterations in some residues of this protein in cotton likely prevented the formation of a TOR-rapamycin-FKBP12 ternary complex, contributing to the observed RAP insensitivity in cotton (Supplemental Fig. 1A).

Given the RAP insensitivity of cotton seedlings, the second generation TOR inhibitor AZD was explored to dissect TOR pathway functions in cotton (Fig. 2A,B). We performed the established functional assay to test the effects of different AZD concentrations on cotton seedling growth. We found that AZD can induce growth retardation of cotton seedlings, and these effects were dose-dependent (as indicated by root length and fresh weight). This suggests that AZD based approach can be used to probe TOR signaling in cotton similar to other plants8,9.

Transcriptome analysis of TOR inhibited cotton. To further dissect TOR functions in cotton, cotton seedlings treated with DMSO (as a control) and AZD were used for RNA-seq analysis. After trimming for quality, 87.77 and 96.96 million RNA-seq clean reads were obtained under the treatment of DMSO and AZD (representing the average of three replicates), respectively (Supplementary Table 2). The corresponding raw datasets and processed files of the RNA-seq data have been deposited in NCBI’s Sequence Read Archive (SRA) under accession number SRX2568809. Approximately 90% of the reads in each of the three replicates were mapped to the cotton genome (Fig. 3A). A total of 6419 differentially expressed genes (DEGs) were found between DMSO and AZD treatment, among these 3767 were up-regulated and 2652 were down-regulated (Fig. 3B). KEGG (Kyoto encyclopedia of genes and genomes) pathway analysis revealed that the up-regulated genes were mainly enriched for several processes including circadian rhythm, fatty acid biosynthesis and phenylpropanoid biosynthesis (Fig. 3C). Besides these, the carbon fixation associated with photosynthesis showed the highly enriched category among the down-regulated genes (Fig. 3D). The plant hormones signal transduction pathway and carbon fixation pathway are consistent with previously reported observations in TOR inhibited Arabidopsis.11 Interestingly, the differentially expressed genes also include some key JA biosynthetic and transduction associated genes, suggesting potential interaction between TOR and JA (Table 2). Furthermore, we also found the altered expression profiles of auxin and abscisic acid (ABA) related genes under TOR inhibition in cotton; these observations are highly consistent with similar treatments in Arabidopsis11 (Supplementary Table 3). As the crosstalk between TOR and ABA

| Protein name | Mammals | Yeast | Arabidopsis | G. hirsutum |
|-------------|---------|------|-------------|-------------|
| SIN1        | ✓       | ✓    | ✓           | ✓           |
| RICTOR      | ✓       | ✓    | ✓           | ✓           |
| PI3Kα       | ✓       | ✓    | ✓           | ✓           |
| PI3Kβ       | ✓       | ✓    | ✓           | ✓           |
| PI3Kγ       | ✓       | ✓    | ✓           | ✓           |
| TSC1        | ✓       | ✓    | ✓           | ✓           |
| TSC2        | ✓       | ✓    | ✓           | ✓           |
| IRS         | ✓       | ✓    | ✓           | ✓           |
| SGK1        | ✓       | ✓    | ✓           | ✓           |
| Akt         | ✓       | ✓    | ✓           | ✓           |
| 4EBP1       | ✓       | ✓    | ✓           | ✓           |
| AMPK        | ✓       | ✓    | ✓           | ✓           |
| LKB1        | ✓       | ✓    | ✓           | ✓           |
| mLST8       | ✓       | ✓    | ✓           | ✓           |
| RAPTOR      | ✓       | ✓    | ✓           | ✓           |
| mTOR        | ✓       | ✓    | ✓           | ✓           |
| FKBP12      | ✓       | ✓    | ✓           | ✓           |
| PDK1        | ✓       | ✓    | ✓           | ✓           |
| S6K         | ✓       | ✓    | ✓           | ✓           |
| RPS6        | ✓       | ✓    | ✓           | ✓           |
| e4        | ✓        | ✓    | ✓           | ✓           |
| E2F3        | ✓       | ✓    | ✓           | ✓           |
| ATPBD3      | ✓       | ✓    | ✓           | ✓           |
| TCTP        | ✓       | ✓    | ✓           | ✓           |

Table 1. The putative components of TOR signaling pathway in cotton.
or auxin signaling pathways have already been previously investigated\textsuperscript{9,29,30}, we focused on JA signaling pathway for further investigation. The expression levels of some randomly-selected genes were verified using QRT-PCR, and the results were consistent with the RNA-Seq analysis (Supplementary Figure 2).

AZD can significantly enhance MeJA-induced growth inhibition on cotton seedlings. Next, we examined whether the potential crosstalk between TOR and JA exists in cotton by applying MeJA (Methyl jasmonate), AZD, and a pairwise combination of MeJA + AZD to cotton seedlings (Fig. 2A,C). The roots of MeJA-treated cotton seedlings turned brown in color, most likely due to the accumulation of polyphenols and anthocyanin. Furthermore, growth inhibition inflicted by the combination of AZD + MeJA in cotton seedlings was more pronounced than the treatments with AZD or MeJA alone. Furthermore, JA level increase induced by TOR inhibition was also observed in cotton seedlings (Fig. 2D). These results indicated that the interplay between TOR and JA may exist in cotton.

MeJA and TOR inhibitors synergistically inhibit plant growth. Due to the lack of mutant collections and difficulties in genetic transformation in cotton, we used the model plant \textit{Arabidopsis} to study the potential interactions between TOR and JA. RAP selectively inhibits TORC1-S6K signaling while AZD is a pan-TOR inhibitor which can directly interact with kinase domain of TOR by competing with ATP. These different properties of RAP and AZD may provide alternative and complementary approaches to investigate the crosstalk between TOR and JA in \textit{Arabidopsis}. WT and BP12-2 \textsuperscript{7} were exposed to MeJA, TOR inhibitors (AZD or RAP) and the pairwise combination of RAP + MeJA or AZD + MeJA (Fig. 4A–E). As expected, WT and BP12-2 seedlings grown on 1/2 MS (Murashige & Skoog) medium supplemented with DMSO, MeJA, RAP and AZD showed phenotypes similar to previous observations.\textsuperscript{7,8,31} However, the combinatorial treatment using MeJA + RAP resulted in BP12-2 but not WT seedlings undergoing enhanced inhibition (regarding fresh weight and root length) (Fig. 4A,B and D). Besides, plants treated with AZD + MeJA showed more pronounced inhibition than with...
AZD or MeJA alone (Fig. 4A,C and E). These results indicated that MeJA and TOR inhibitors may synergistically inhibit plant growth.

To quantitatively measure the synergism generated by the interaction between TOR inhibitors and MeJA, CI (Combination index) values were used in this study. First, to further examine whether the synergism can be generated by the combination of TOR inhibitors (RAP or AZD) with MeJA, we investigated the G150 (50% growth inhibitory dose) values of each drug alone and pairwise combinations in BP12-2 seedlings (Supplementary Table 4). The G150 values of MeJA, RAP and AZD were 10, 0.35 and 1 µM, respectively. However, their G150 values were significantly reduced when BP12-2 were subjected to MeJA + RAP or MeJA + AZD treatments, implying that the potential synergistic effects can be generated by combining MeJA and TOR inhibitors. Next, computer-simulated Fa (Affected fraction)-CI curves were assessed by using the CompuSyn software.
Figure 3. RNA-Seq analysis of the *G. hirsutum* seedlings exposed to AZD. (A) Proportions of clean reads of unmapped, mapped to unique genes and mapped to multiple genes, which were plotted by three replicates of DMSO and three replicates of AZD on the horizontal axis. (B) Number of up-regulated and down-regulated DEGs. (C) Significantly enriched KEGG pathway for up-regulated DEGs. (D) Significantly enriched KEGG pathway for down-regulated DEGs. The pathways in (B) and (C) were ranked by their significance. DEGs: Differentially Expressed Genes. KEGG: Kyoto Encyclopedia of Genes and Genomes.
results confirmed that MeJA and TOR inhibitors (AZD or RAP) could synergistically inhibit plant growth. In cotton, we speculated that TOR inhibition also influenced the endogenous JA content in lines (Supplementary Figure 3A).

MeJA and TOR inhibitors had a synergistic effect on the mRNA levels of these genes in BP12-2 transgenic more dramatic manner than those treated with single drug alone. The CI curves of two selected genes suggested treated with the combination of TOR inhibitor and MeJA, relative mRNA levels of these genes were changed in a (Fig. 5A–F and Supplementary Figure 3B). In both BP12-2 transgenic lines (Fig. 5A–F) and WT herefore, we measured the endogenous JA levels in biosynthetic genes the major JA biosynthetic and responsive genes. The transcription levels of six genes including the major JA talk between TOR and JA signaling pathway, we examined the effect of TOR inhibition on the mRNA levels of with the QRT-PCR data shown above. Taken together, our data suggest that TOR has a negative effect on JA bio-

TOR negatively influences JA biosynthesis and plant response to JA. To further dissect the crosstalk between TOR and JA signaling pathway, we examined the effect of TOR inhibition on the mRNA levels of the major JA biosynthetic and responsive genes. The transcription levels of six genes including the major JA biosynthetic genes AOC1 (Allene oxide cyclase1) and OPR3 (OPDA reductase3)22,23 that have a close relationship with JA content in plants, repressors of JA signaling pathway JAZ1 and JAZ217–19 whose transcript levels showed significant changes in the RNA-seq data of TOR-inhibited Arabidopsis seedlings31; cell cycle related gene CYCB1;1 (Cyclin-dependent protein kinase1;1)34 which is greatly affected by JA signaling pathway; and the bHLH transcription factor MYC235 which functions as a regulator of JA-responsive gene expression were analyzed (Fig. 5A–F and Supplementary Figure 3B). In both BP12-2 transgenic lines (Fig. 5A–F) and WT Arabidopsis (Supplementary Figure 3B), TOR inhibition and MeJA treatments resulted in a decrease in the relative transcript levels of CYCB1;1, but led to an increase in the expression levels of the other five genes. Besides, when plants were treated with the combination of TOR inhibitor and MeJA, relative mRNA levels of these genes were changed in a more dramatic manner than those treated with single drug alone. The CI curves of two selected genes suggested that MeJA and TOR inhibitors had a synergistic effect on the mRNA levels of these genes in BP12-2 transgenic lines (Supplementary Figure 3A).

Since TOR suppression increased the relative transcript levels of JA biosynthetic genes (Table 2) and JA levels (Fig. 2D) in cotton, we speculated that TOR inhibition also influenced the endogenous JA content in Arabidopsis. Therefore, we measured the endogenous JA levels in Arabidopsis (Fig. 5G). Consistently, our results showed that suppression of TOR resulted in the significant increase in JA production in Arabidopsis, which was in agreement with the QRT-PCR data shown above. Taken together, our data suggest that TOR has a negative effect on JA biosynthesis and its response.

Sensitivity of JA synthesis and signaling mutants to AZD. Next, we examined whether the mutants jar1, coi1-2, jaz10 and myc2-2 deficient in crucial steps of JA synthesis and transduction are affected by AZD treatments (Fig. 6). Interestingly, the results showed that jar1, coi1-2 and myc2-2 were insensitive to AZD compared with WT, as indicated by root length and fresh weight data (Fig. 6B and D). In contrast, the transcriptional repressor mutant jaz10 was sensitive to AZD (Fig. 6A,B). Moreover, the finding that coi1-2 was insensitive to AZD prompted us to generate COII overexpression transgenic lines (Fig. 6C,D). COII-overexpression transgenic lines were generated by introducing P35S::COII-HA into WT Arabidopsis. These COII-overexpression transgenic lines showed AZD-hypersensitivity phenotypes including slower growth, smaller leaf size and shorter root length than

| Arabidopsis homologs | Gene ID | Log2(Fold change) | P-adjusted | Annotation                  |
|----------------------|---------|-------------------|------------|-----------------------------|
| AOS                  | Gh_D06G089 | 4.62              | 5.0E-04    | allene oxide synthase        |
|                      | Gh_D06G087 | 2.41              | 5.0E-04    | allene oxide synthase        |
|                      | Gh_A06G0111 | 3.82             | 5.0E-04    | allene oxide synthase        |
| OPR3                 | Gh_D05G0339 | 1.23634         | 5.0E-04    | oxophytodienoate reductase 3 |
| LOX                  | Gh_A08G1863 | 2.39364         | 5.0E-04    | lipoxygenase 1               |
|                      | Gh_D13G1129 | 1.50253         | 5.0E-04    | lipoxygenase 1               |
|                      | Gh_D08G2224 | 2.52251         | 5.0E-04    | lipoxygenase 1               |
|                      | Gh_A13G0888 | 1.44615         | 5.0E-04    | lipoxygenase 1               |
| ACX1                 | Gh_A11G2972 | 1.04194         | 5.00E-04   | acyl-CoA oxidase 1           |
|                      | Gh_A12G1964 | 0.861585        | 1.36E-03   | acyl-CoA oxidase 1           |
|                      | Gh_D11G0264 | 1.43708         | 5.0E-04    | acyl-CoA oxidase 1           |
|                      | Gh_D12G2143 | 0.951985        | 5.0E-04    | acyl-CoA oxidase 1           |
| JMT                  | Gh_A10G0863 | 2.33821         | 1.97E-02   | jasmonate O-methyltransferase|
|                      | Gh_A12G0585 | 3.40333         | 5.80E-03   | jasmonate O-methyltransferase|
|                      | Gh_A12G0584 | 2.53434         | 1.76E-03   | jasmonate O-methyltransferase|
| CUL                  | Gh_D01G2280 | 0.710028        | 1.16E-02   | cullin 1                     |
| JAZ                  | Gh_A12G2441 | 1.31113         | 7.53E-03   | jasmonate-zim-domain protein 3|
|                      | Gh_A06G0705 | 1.70817         | 1.93E-02   | jasmonate-zim-domain protein 1|
|                      | Gh_A08G2199 | 2.22148         | 2.73E-02   | jasmonate-zim-domain protein 1|
|                      | Gh_D08G2564 | 3.20572         | 1.88E-02   | jasmonate-zim-domain protein 1|
|                      | Gh_D12G2567 | 0.745434        | 1.03E-02   | jasmonate-zim-domain protein 3|
| TOPLESS              | Gh_A08G1965 | 0.774929        | 6.36E-03   | TOPLESS protein              |
|                      | Gh_A05G1044 | 1.60034         | 5.0E-04    | TOPLESS protein              |
| MYC2                 | Gh_A09G2341 | 1.44412         | 4.52E-03   | Transcription factor MYC2    |
Figure 4. TOR inhibitors and MeJA synergistically inhibit the growth of Arabidopsis seedlings. (A) Phenotypes of 10-day-old WT and BP12-2 seedlings exposed to DMSO, 10 μM MeJA, 0.35 μM RAP, 1 μM AZD and the combination of MeJA + RAP or MeJA + AZD. Fresh weight (B) and root length (D) of BP12-2 seedlings treated with DMSO, 0.35 μM RAP, 10 μM MeJA and the combination of MeJA + RAP. Fresh weight (C) and root length (E) of WT seedlings treated with DMSO, 1 μM AZD, 10 μM MeJA and the combination of MeJA + AZD. Each graph in (B–E) represents the average of 10 seedlings that were conducted in triplicate. Error bars represent ± SD (n = 3). Asterisks indicate significant differences in root length or fresh weight between DMSO and indicated treatments at **p < 0.01 (Student’s t-test). (F–G) Fa–CI curve shows synergism (CI < 1) between MeJA and TOR inhibitors.
WT, which displayed the opposite phenotype observed in coi1-2 (Fig. 6C). These independent lines of evidence further revealed the crosstalk between TOR and JA signaling pathways at multiple sites and levels.

**GhTIFY genes are involved in the crosstalk between TOR and JA.** The above results indicated that TOR has a negative effect on JA biosynthesis and its signal transduction. JAZs, the subfamily of the TIFY family, are well-established transcriptional repressors of JA signaling pathway[^17^–^19^]. It is possible that the growth defects caused by TOR suppression can be rescued by GhJAZ overexpression. To verify this hypothesis, we next examined the interactions between cotton JAZ/TIFY and TOR by overexpressing cotton JAZ/TIFY genes in *Arabidopsis* BP12-2 background. Twenty-one putative TIFY genes in *G. hirsutum* were cloned and named following the existing nomenclature system used in *Arabidopsis* (Supplementary Table 5). Phylogenetic construction and motif of these genes were shown in Supplementary Figure 4A. Among the thirteen JAZ genes, two JAZ overexpression transgenic lines *GhTIFY7b* (JAZ7) and *GhTIFY10a* (JAZ10) showed a relative better growth condition under TOR inhibition than other transgenic lines, thus *GhTIFY7b* and *GhTIFY10a* were selected for further analysis (Fig. 7 and Supplementary Figure 4B,C). We found that these transgenic lines were partly insensitive to TOR inhibitors compared with BP12-2, indicating that GhTIFY overexpression can partly rescue the growth defects of TOR suppression (Fig. 7A,B). Besides, when cotton seedlings were exposed to TOR inhibitors and MeJA, the relative transcript level changes of these two genes were consistent with the results in *Arabidopsis* (Fig. 7C).

In *Arabidopsis*, JAZ proteins are degraded by 26S proteasome when plants respond to JA[^17^–^19^]. To verify whether MeJA and TOR inhibitors could induce the degradation of GhTIFY proteins, 35S-GhTIFY-GUS transgenic plants (*GhTIFY7b* and *GhTIFY10a*) were generated and exposed to MeJA and TOR inhibitors (Fig. 7D–F and Supplementary Figure 4C). Consistent with the expectation, MeJA treatment induced significant decrease in GUS activity in these transgenic lines. Interestingly, RAP and AZD can mimic MeJA to promote the degradation of GhTIFY proteins.

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**Figure 5.** TOR inhibition influences JA biosynthesis and response in plants. 6-day-old BP12-2 seedlings were treated with DMSO, 0.35µM RAP, 10µM MeJA and the combination of MeJA + RAP for 24 h. Relative expression levels of JA biosynthetic genes *AtAOC1* (A) and *AtOPR3* (B), and JA responsive genes *AtJAZ1* (C), *AtJAZ7* (D), *AtCYCB1;1* (E) and *AtMYC2* (F) were examined. The expression levels of these genes in DMSO were set to 1. (G) JA contents in BP12-2 and WT seedlings exposed to TOR inhibitors. Each independent experiment contains 10 plants per treatment. JA is quantified once for the pool of 10 plants. Error bars represent ± SD (n = 3). Three biological replicates were analyzed with similar results. Asterisks (**p < 0.01**) and different letters indicate significant differences from the control.
of GhTIFY-GUS protein in Arabidopsis. These observations further confirmed that TOR is involved in the JA signaling in plants.

Discussion

Most examined heterotrophic organisms contain two distinct TOR complexes: TORC1 and TORC2, and TOR signaling pathway has been well documented in several model organisms\(^1\)-\(^4\). However, little information is known about TOR signaling pathway in cotton. Based on the recently released genome database of cotton (http://cgp.genomics.org.cn/), we identified the putative components of TOR complexes, the upstream and downstream factors of TOR signaling pathway for the first time. Unlike the FATC domain’s essential functions for TOR protein kinase in yeast and animals\(^3\), in Arabidopsis, this domain appears dispensable and no clear phenotype can be observed with its absence\(^3\). We found that FATC domain only exists in GhTOR1 but not in GhTOR2. The detailed function of this domain in cotton remains to be understood. Moreover, the significant expansion of TORC1/S6K/RPS6 in cotton genome implies that this signaling cascade may be the mainstay of TOR signaling pathway in cotton. These bioinformatic investigations uncovered an expansion and diversification of TOR genes and the canonical TOR components in cotton, which might point to new additional functions for TOR signaling.

Rapamycin and AZD were employed to probe TOR signaling in this study. They both target TOR protein, but the modes of action, molecular mechanism, inhibitory spectrum and drug potency of rapamycin are quite different from that of AZD. Rapamycin is the first generation TOR inhibitor and specifically inhibit TORC1-S6K signaling branch, whereas AZD is the second generation of TOR inhibitor and can suppress most outputs of TORC1 and TORC2. Since TOR has many upstream signaling inputs and downstream signaling outputs in eukaryotic organisms and different signaling cascades operated through these have different functions, both selective TOR inhibitor (rapamycin) and pan-TOR inhibitor (AZD) based approaches are useful for defining TOR signaling in plants. Rapamycin/FKBP12/TOR ternary complex provides a conditional and highly inducible selective system to decipher TORC1-S6K-RPS6 signaling. Considering TORC1-S6K-RPS6 is the mainstay of cotton TOR signaling,
rapamycin should be a very important tool to dissect TOR signaling if GhFKBP12 is able to bridge the interaction between RAP and TOR. However, our observations suggest that GhFKBP12 is defective to interact with RAP and not effective to produce TOR inhibition. As an alternative, we have successfully employed AZD to probe TOR signaling in cotton; and the Arabidopsis BP12-2 line was successfully applied to detect the functions of GhTIFYs in TOR signaling by using RAP. These results demonstrate that RAP and AZD have many overlapping and complementary advantages for TOR signaling research in cotton and Arabidopsis.

Cotton RNA-seq analysis was employed to elucidate the function of TOR signaling. The RNA-Seq analysis show that TOR inhibition result in changes in many metabolic processes, which further implies that TOR plays a key role in cotton growth and development. Besides, plant hormone signal transduction, JA-related genes were significantly altered in TOR inhibited cotton seedlings (Fig. 3C and Table 2). In Arabidopsis, the expression profiles of representative JA related genes were also altered under TOR suppression by AZD. These results indicate the potential interaction between JA and TOR in cotton. The precise molecular mechanism that mediates their crosstalk remains to be elucidated. In Arabidopsis, TOR inhibition can be partially rescued by a mutation in ABI4 (ABA Insensitive 4), which encodes an ABA-regulated AP2 domain transcription factor. In cotton, there were two putative homologs of ABI4 (GotAD_22335 and GotAD_43460) whose protein sequences show a relatively low identity (46% and 47%) with that of Arabidopsis. In the DEGs of our cotton RNA-seq data, we didn’t find these two putative homologs, potentially either due to the loss of the evolutionary functional conservation for these genes or they do not represent the true orthologs. Besides, the transcript levels of the other key node genes of ABA signaling pathway were changed in cotton for instance PYL (PYRABACTIN resistance 1-like), ABF (Abscisic acid responsive elements-binding factor) and PP2C (Type 2C protein phosphatases) family.

Figure 7. Effect of TOR inhibitors on GhTIFY overexpression lines and GhTIFY protein degradation. (A) GhTIFY overexpression lines were partially insensitive to TOR inhibitors. GhTIFY7b and GhTIFY10a overexpression transgenic lines were in Arabidopsis BP12-2 background and exposed to DMSO and 0.35 µM RAP + 1 µM AZD for ten days. Scale bar = 1 cm. (B) Root length and fresh weights of GhTIFY overexpression lines in (A). Each graph represents the average of 10 seedlings that were conducted in triplicate. (C) Analysis of GhTIFY7b and GhTIFY10a expression levels in cotton seedlings exposed to DMSO, 5 µM AZD, 10 µM MeJa and the combination of AZD + MeJa. The expression levels of these genes in DMSO were set to 1. (D,E) 35S-GhTIFY10a-GUS OE3 overexpression transgenic lines were treated with DMSO, MeJa (10 µM), or RAP (0.35 µM) + AZD (1 µM) for 48 h. The leaves (D) and roots (E) were used to perform the GUS staining assays. (F) GUS activity quantification of 35S-GhTIFY10a-GUS OE3. In (C) and (F), Error bars represent ± SD (n = 3). Different letters indicate different significant differences from the control.
protein (Supplementary Table S3). Repression of ABA signaling is a potential mechanism to enhance JA signaling indirectly.

Interestingly, AZD treatment mimics the growth defects generated by MeJA treatment in cotton seedlings. Importantly, the endogenous levels of JA were significantly increased when cotton seedlings were subjected to AZD, along with the transcriptional changes of JA biosynthetic and responsive genes in both cotton and Arabidopsis. TOR inhibition not only altered the expression levels of JA-responsive genes, but also influenced the expression of genes involved in JA biosynthesis. These results suggest important functions for broader crosstalk between JA and TOR pathways in cotton. To test this hypothesis, we performed the pharmacological analysis in cotton by combining AZD with MeJA, and stronger inhibitory effects were observed than that of single drug treatment (Fig. 2). Furthermore, using a statistical analysis software\(^{10,37}\), we found the growth-inhibition and gene-expression effects of these treatments acted synergistically when Arabidopsis were subjected to the combined treatment of RAP + MeJA or AZD + MeJA. These independent lines of evidences strongly suggest the possibility of the interaction between JA and TOR signaling. To further confirm these results, using genetic approaches, mutants deficient in crucial steps of JA synthesis and perception including jar1, coi1-2, jaz10 and myc2-2 were employed to examine TOR signaling. Our results also show that the loss of function mutant jar1, coi1-2 and myc2-2 was insensitive to AZD, whereas jaz10 and COI1 overexpression result in a phenotype, which was a complete opposite to that of the other mutants (Fig. 6). The JAZ protein, which belongs to a subfamily of the TIFY family, is a transcriptional repressor of JA\(^{17-19}\). When plants respond to JA, JAZ protein is ubiquitinated and subsequently degraded by the 26S proteasome. Because the understanding of the plant-specific TIFY family in cotton is limited, we identified 21 putative TIFY genes in cotton, analyzed their sequences (Supplementary Figure 4A), and investigated their functions in the model plant Arabidopsis (Fig. 7). The results from these studies show that over-expression of the TIFY genes could partially compensate the growth inhibition caused by TOR inhibitors, and TOR inhibition could also induce the degradation of the TIFY protein. These results suggest that genetic disruptions of JA signaling reduce the effects of TOR inhibition by using the coi1-2, jar1, and myc2-2 mutants and strong expression of putative JAZ repressors from cotton, while potentiating JA signaling through over-expression of COI1 and using jaz10 mutant enhances the growth retarding effects of TOR inhibition.

Based on the findings of the present study, we propose a working model for the interaction between TOR and JA signaling pathways in plants (Supplementary Figure 5). Since TOR signaling pathway plays a key role in plant growth and development, suppression of TOR creates a stress for plants. As JA is an important regulator of plant stress response, TOR inhibition triggers plant stress response by promoting JA biosynthesis and activation of JA signaling. When TOR was inhibited, JA biosynthesis renders JAZ degradation. The genetic evidences presented in this study provide crucial insights into the cross-talk between TOR and JA signaling pathways at multiple levels including JAR1, COI1, MYC2 and JAZ.

Methods

Materials and growth conditions. Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used as the wild-type plants in this study. The transgenic Arabidopsis BP12-2 was generated in a previous study\(^7\). The Arabidopsis mutant lines jar1, coi1-2, myc2-2 and jaz10 were kindly provided by Dr. Chuanyou Li of the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences. Arabidopsis seeds were surface-sterilized with 70% ethanol for 3 min; followed by 10% bleach containing 0.3% Tween-20 for 10 min; and then rinsed by sterile water for three times. The sterilized seeds were suspended in 0.1% sterile agarose and kept at 4 °C for two days, and then germinated on 1/2 MS (Murashige and skoog) medium plates supplemented with reagents as indicated in Figures. All Arabidopsis were grown at 22 °C under long-light conditions (16 h light/8 h dark). Wild-type G. hirsutum (CCRI24) is used in this study. Cotton seeds were surface-sterilized with 0.1% mercuric chloride for 5 min, rinsed with sterile water. The sterilized seeds were sown in MS medium containing different solutions and 1% agar, and grown in a growth chamber at 25 °C under 16 h light/8 h dark condition. Plant growth showed no difference between MS and MS + DMSO medium, and all drugs were dissolved in DMSO, MS + DMSO medium was set as a control in this study.

Combination index (CI) value measurement. Combination index (CI) values were used to quantitatively measure the interaction between MeJA and TOR inhibitors\(^10\). The degree of reagent interaction is based on synergism (CI < 1), additive effect (CI = 1), or antagonism (CI > 1)\(^37\). 6-day-old BP12-2 seedlings were incubated in 48-well plates containing 1/2 MS liquid medium supplemented with DMSO (as a control), different concentrations of MeJA and RAP, or a combination of MeJA + RAP for 4 d in a growth chamber with a light intensity of 100 μmol/m²/s at 22 °C. Fresh weight of 6- and 10-day-old seedlings in every well was measured to calculate growth inhibition. Experiments were repeated three times. Percentage of growth value was calculated by using the fresh weight of 6-day-old seedlings (W6), DMSO-treated control plants (C), and drug-treated plants (D) as follows: [(D-W6)/(C-W6)] × 100. The 50% growth inhibitory dose (GI50) and CI values were calculated using the CompuSyn software program\(^46\). Fa (Affected fraction) represented the fraction of plants’ fresh weight affected by the reagent. The values of Fa indicated a growth inhibition value and were calculated according to the software instructions as follows: [(100-%growth value)/100].

Genome-wide identification of G. hirsutum TOR signaling pathway components, FKBP12 and TIFY family. Arabidopsis TOR and FKBP12 protein sequences were used as queries searching for cotton homologs in the cotton genome database (http://cgp.genomics.org.cn)\(^25\). Sequences of humans and Arabidopsis TOR signaling pathways components were used to identify cotton homologs in the same database. The 21 G. hirsutum TIFY genes were identified by aligning the TIFY domain sequences with the cotton genome (http://cgp.genomics.org.cn/). E-value (1e–10) was set to obtain the final TIFY proteins. Phylogenetic tree was constructed...
by MEGA4.0 using the neighbor-joining method39,40. Motif analysis was performed using MEME (http://meme.sdsc.edu/meme)41.

**Plasmid constructions and generation of transgenic plants.** Full-length CDS were amplified by PCR using the TransStar Taq polymerase Mix kit (TRANSGEN) following the manufacturer’s instructions. The list of primers is shown in Supplementary Table 6. The recombinant plasmids were generated as described previously7. The transgenic lines were generated by Agrobacterium-mediated transformation using the floral dip method42. Transgenic plants seeds were germinated on 1/2 MS medium containing 50 mg/L kanamycin and confirmed by PCR analysis. The T3 homozygous plants were used in the experiments.

**Quantitative real-time PCR.** Total RNA was isolated from cotton or Arabidopsis seedlings as indicated in Figures using the RNaprep Pure Plant kit (TIANGEN). PrimeScript RT reagent kit (TAKARA) was used to generate first-strand cDNA products. The CFX96 real-time PCR system (BIO-RAD) was used to measure relative transcript expression levels by using an SYBR Premix Ex Taq II (TAKARA) kit. Primers used for qRT-PCR were listed in Supplementary Table 6. Expression levels of target genes were normalized to ACTIN2 and GHISTONE3 in Arabidopsis and cotton, respectively. The relative quantitative method (2^−ΔΔCT) was used to calculate the quantitative variation43. The data were expressed as the mean ± SD of three independent experiments. At least 5–10 plants of every treatment were sampled for each independent biological replicate.

**GUS staining and GUS activity quantification.** X-Gluc was used in GUS staining following a previously reported procedure44. Images of the representative lines were captured using a stereomicroscope (OLYMPUS MVX10). A MarkerGene™ β-glucuronidase (GUS) reporter gene activity detection kit (Marker Gene Technologies) was used to quantify GUS activity. The Bradford assay was used for total protein quantification45. Three biological experiments, each consisting of 30 plants per treatment were measured.

**JA content measurements.** Two-week-old Arabidopsis or six-day-old cotton seedlings were transferred to 1/2 MS medium containing DMSO or various TOR inhibitors for 48 hours. Each treated Arabidopsis or cotton seedling was harvested for measurements of JA contents. JA was extracted following the exact extraction procedure46. JA extraction was quantified by GC-MS (Gas Chromatography-Mass Spectrometer) methods as previously reported47. Three biological experiments, each consisting of 10 plants per treatment were measured. JA was quantified once for the pool of 10 plants.

**Transcriptome analysis.** 4-d-old cotton seedlings were transferred into MS medium containing DMSO and 5 µM AZD for 24 h, respectively. Total RNA was isolated as described above. Three biological repeats were employed to transcriptome analysis. Sequencing libraries were generated by NEB Next Ultra™ Directional RNA Library Prep Kit for Illumina (New England Biolabs) following the manufacturer’s recommendations. An Illumina Hiseq 4000 platform was used to sequence these libraries, and 100bp paired-end reads were generated. TopHat2 was used to map these reads to the G. hirsutum transcripts with the help of genome annotation48,49. The mapped reads were assembled by CuppLinks50. Differentially expressed genes (DEGs) were identified using Cuffdiff51. To investigate the DEGs between the control and treated libraries, fold change of each gene was calculated as the ratio of read counts in the treated libraries to the read counts in the control library followed by transformation of log2. Genes with a P-value ≤ 0.8 and a |log2Ratio| value ≥ 1 were identified as significant differentially expressed genes. KOBAS software was used to test the enrichment of DEGs in KEGG pathways52,53. KEGG enrichment analysis was obtained with a P-value of < 0.05.

**Statistical analysis.** SPSS 20.0 software (IBM, Armonk, NY, USA) was used to perform all statistical analysis of data. The data represent the mean ± SD of n = 3 independent experiments. ANOVA analysis and a Tukey-Kramer multiple comparison test was used for data analysis. P < 0.05 was considered as statistically significant.

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Author Contributions

F.L., M.R. and Y.S. designed the experiments. Y.S., G.Z., X.Z. and L.L. performed the experiments. F.X., F.Z., C.Z., and Z.Y. analyzed the data. M.R., Y.S., and R.D. wrote the manuscript.

Additional Information

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