# New Insights into Roles of Cell Wall Invertase in Early Seed Development Revealed by Comprehensive Spatial and Temporal Expression Patterns of *GhCWIN1* in Cotton[1][W][OA]

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Despite substantial evidence on the essential roles of cell wall invertase (CWIN) in seed filling, it remains largely unknown how CWIN exerts its regulation early in seed development, a critical stage that sets yield potential. To fill this knowledge gap, we systematically examined the spatial and temporal expression patterns of a major CWIN gene, *GhCWIN1*, in cotton (*Gossypium hirsutum*) seeds from prefertilization to prestorage phase. *GhCWIN1* messenger RNA was abundant at the innermost seed coat cell layer at 5 d after anthesis but became undetectable at 10 d after anthesis, at the onset of its differentiation into transfer cells characterized by wall ingrowths, suggesting that CWIN may negatively regulate transfer cell differentiation. Within the filial tissues, *GhCWIN1* transcript was detected in endosperm cells undergoing nuclear division but not in those cells at the cellularization stage, with similar results observed in Arabidopsis (*Arabidopsis thaliana*) endosperm for CWIN, *AtCWIN4*. These findings indicate a function of CWIN in nuclear division but not cell wall biosynthesis in endosperm, contrasting to the role proposed for sucrose synthase (*Sus*). Further analyses revealed a preferential expression pattern of *GhCWIN1* and *AtCWIN4* in the provascular region of the torpedo embryos in cotton and Arabidopsis seed, respectively, indicating a role of CWIN in vascular initiation. Together, these novel findings provide insights into the roles of CWIN in regulating early seed development spatially and temporally. By comparing with previous studies on *Sus* expression and in conjunction with the expression of other related genes, we propose models of CWIN- and *Sus*-mediated regulation of early seed development.

Suc is the primary carbon source translocated through phloem from photosynthetic leaves to nonphotosynthetic sinks such as developing seed. Prior to its use for metabolism and biosynthesis, Suc must be degraded into hexoses, by either Suc synthase (*Sus*; EC 2.4.1.13) or invertase ($\beta$-fructosidase; EC 3.2.1.26). *Sus* cleaves Suc into UDP-Glc and Fru and is largely involved in cell wall and starch biosynthesis (Chourey et al., 1998; Brill et al., 2011) and maintaining sink strength (Pozueta-Romero et al., 1999; Xu et al., 2012). Invertase, on the other hand, irreversibly hydrolyzes Suc into Fru and Glc and plays vital roles in plant development and stress responses (Ruan et al., 2010; Li et al., 2012).

Invertase can be classified into three subgroups as cell wall invertases (CWINs), cytoplasmic invertases, and vacuolar invertases (VINs; Roitsch and González, 2004). Different from the other two isoenzymes, CWINs are insoluble proteins ionically bound to the cell wall, cleaving Suc in the apoplasm. The resultant hexoses are often transported into cells across plasma membranes by hexose transporters. Therefore, CWINs are central to phloem unloading and carbon partitioning, especially in cellular sites lacking symplasmic (plasmodesmal) connections (Patrick and Offler, 2001; Weschke et al., 2003). A positive correlation between CWIN activity and seed development has been observed in maize (*Zea mays*; Cheng et al., 1996; Vilhar et al., 2002; Chourey et al., 2006), faba bean (*Vicia faba*; Weber et al., 1995, 1996), barley (*Hordeum vulgare*; Weschke et al., 2003; Sreenivasulu et al., 2004), rice (*Oryza sativa*; Hirose et al., 2002; Wang et al., 2008), and tomato (*Solanum lycopersicum*; Zanor et al., 2009). With an exception in tobacco (*Nicotiana tabacum*; Tomlinson et al., 2004), high and low Glc-to-Suc ratios generally favor cell division or promote cell differentiation, respectively, during seed development (for review, see Weber et al., 2005). Consistently, high CWIN activities correlate with high Glc level and mitotic activity in faba bean embryo (Weber et al., 1996, 1997) and maize endosperm (Cheng et al., 1996; Vilhar et al., 2002). Loss of CWIN expression reduces seed filling in maize (Cheng et al., 1996) and rice (Wang et al., 2008), whereas elevation of CWIN activity by silencing its inhibitor increases seed size in tomato (Jin et al., 2009), demonstrating the critical role of CWIN in seed development.

The progress described above, however, mainly focuses on the roles of CWIN in seed filling from

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prestorage to storage phases; it remains largely unknown how CWIN exerts its potential role early in seed development, a critical stage that sets yield potential. Seed set is determined during fertilization and within several days post fertilization and is characterized by intensive cell division in filial tissues, which may be controlled by CWIN-mediated Glc signaling (Ruan et al., 2012). Moreover, as in mammals, early embryo development is highly sensitive to biotic or abiotic stresses leading to abnormal development or even abortion, hence irreversible yield loss (Ruan et al., 2012). Recent studies show that reduction of invertase activity is likely a major factor causing early abortion of maize grain under drought (Boyer and McLaughlin, 2007) and tomato fruit under heat stress (Li et al., 2012). Thus, elucidation of how CWIN regulates early seed and fruit development will not only advance understanding of plant reproductive biology but could also lead to new ways to improve crop yield and tolerance to stress.

As the first step toward filling this knowledge gap, we systematically examined the spatial and temporal expression of a CWIN, *GhCWIN1*, in cotton (*Gossypium hirsutum*) seeds from prefertilization to prestorage phases. In cotton seed, Suc is unloaded from phloem in outer seed coat and delivered outward to seed epidermis for fiber growth and inward to filial tissues (Ruan et al., 1997). At approximately 10 d after anthesis (DAA), the innermost layer of cotton seed coat develops into transfer cells (TCs) with invaginated cell wall ingrowths (WIs), which facilitate the efflux of nutrient to developing endosperm and embryo (Pugh et al., 2010). These features render cotton seed an ideal system to explore the role of CWIN in early seed development. To achieve this, we cloned a major CWIN complementary DNA (cDNA), *GhCWIN1*, from young cotton seeds. By examining its spatial and temporal expression in cotton seeds from prefertilization to prestorage phases, we made several novel observations on the dynamics of *GhCWIN1* expression in a developmental context that, together with studies on *AtCWIN4* in Arabidopsis (*Arabidopsis thaliana*), indicate new roles of CWIN in early seed development, particularly in regulating endosperm nuclear division, embryonic provascular formation, and TC differentiation.

## RESULTS

### Cloning of a Cotton CWIN cDNA That Is Highly Expressed in Developing Seed

To clone CWINs that are expressed in developing cotton seed, we searched a cotton EST database (http://www.cottondb.org) and GenBank (http://www.ncbi.nlm.nih.gov/genbank/), which identified a 633-bp partial-length cDNA clone (GenBank accession no. AI725433) encoding a 210-amino acid peptide highly homologous to the corresponding regions of acid invertases from other plants. By using reverse transcription (RT)-PCR, coupled with 5′ and 3′ RACE, a 2,075-bp cDNA clone was isolated from 5-DAA cotton seed and named *GhCWIN1*.

*GhCWIN1* contains a full-length open reading frame (ORF) of 1,764 bp, encoding 587 amino acids, with a predicted signal sequence cleavage site between residues 19 and 20 (Supplemental Fig. S1). TBLASTN and BLASTP searches showed a much higher similarity of GhCWIN1 with plant CWINs than that of VINs. For example, GhCWIN1 shared 60% and 57% amino acid identities with CWIN from sugar beet (*Beta vulgaris*) and grape (*Vitis vinifera*; GenBank accession nos. CAD19323 and AAT09980, respectively) but only 37% with VIN from cotton (GenBank accession no. FJ915120). Alignment analyses revealed that GhCWIN1 exhibited 13 regions conserved in known acid invertases, including the characteristic $\beta$-fructosidase motif (NDPD/NG; Ji et al., 2005). Importantly, GhCWIN1 contained Pro in the Cys catalytic domain (WEC<u>P</u>D), diagnostic to CWIN and displayed five distinctive amino acids (Supplemental Fig. S1) that are highly conserved in CWINs but are different from those in VINs (Ji et al., 2005). Consistently, phylogenetic analyses revealed that GhCWIN1 was clustered in the plant CWIN group, which is evolutionarily close to plant VINs but distant from plant cytoplasmic invertases, yeast invertase, and bacterial invertases; Supplemental Fig. S2). Furthermore, prediction analyses using three programs, PSORT (http://wolfpsort.org/), Target P (http://www.cbs.dtu.dk/services/TargetP/), and Signal P (http://www.cbs.dtu.dk/services/SignalP/), unanimously assign an apoplasmic targeting of GhCWIN1 with high probability of 81.4%, 92.3%, and 95.1%, respectively. Collectively, the above analyses indicate strongly that *GhCWIN1* encodes a CWIN.

Due to the unavailability of the cotton genome sequence, it is not possible to determine the exact size of the CWIN gene family in cotton. Nevertheless, our bioinformatic and gene expression analyses coupled with the enzyme assay described below indicate that *GhCWIN1* is a major CWIN member expressed in cotton seed. Here, BLASTN search of the cotton EST database (http://www.cottondb.org) with the *GhCWIN1* ORF sequence identified 57 BLAST hits, which could be assembled into nine contigs. Among those, four assembled sequences showed identities larger than 96%, suggesting that they may well be *GhCWIN1* alleles, suggesting that *GhCWIN1* is a highly expressed CWIN isoform in cotton. Notably, all of these allele sequence-assembling ESTs were derived from cotton fiber and ovule/seed in early stages (−3 to 10 DAA), indicating important roles of GhCWIN1 in early cotton seed development. Consistently, RT-PCR experiments revealed that *GhCWIN1* was highly expressed in developing seed and root but weakly in stem, cotyledons, and source leaves (Fig. 1A). Developmentally, *GhCWIN1* transcript levels were high in elongating fiber and seed at an early stage at 5 DAA, decreased at 10 and 15 DAA, and became barely detectable in the storage phase at 20 DAA (Fig. 1B; Supplemental Fig. S3), indicating potential roles of GhCWIN1 in early seed

development. Similar to the *GhCWIN1* transcript level (Fig. 1B), the CWIN activities in fiber and seed coat also decreased from 10 to 20 DAA (Fig. 2). Moreover, compared with that in fiber and seed coat, CWIN activity in filial tissue was much lower (Fig. 2), similar to that observed at the mRNA level of *GhCWIN1* (Fig. 1B).

In planta CWIN activity is capped by its inhibitory protein (Jin et al., 2009). Extensive database searches identified *GhINH1* (GenBank accession no. AI725842) as a major putative invertase inhibitor expressed in cotton seed based on the number of ESTs detected. GhINH1 is considered as a CWIN inhibitor, since it was unanimously predicted to target extracellular space analyzed with four different programs, WoLF PSORT (http://wolfpsort.org/), TargetP (http://www.cbs.dtu.dk/services/TargetP/), Protein Prowler (http://pprowler.imb.uq.edu.au), and CELLO Prediction (http://cello.life.nctu.edu.tw/). As revealed by semiquantitative RT-PCR (Supplemental Fig. S3), *GhINH1* was constantly but weakly expressed in 5-DAA seed and 10- to 15-DAA filial tissues, suggesting that CWIN activity may be limited by GhINH1 during seed development.

The bioinformatic analyses and the overall pattern similarity between changes of *GhCWIN1* transcript and CWIN activity (Figs. 1 and 2) suggest that GhCWIN1 is likely a major contributor to CWIN activity in cotton seed. Noteworthy is that CWIN activities remained at relatively high levels in the cotton ovule/seed from −1 to 5 DAA (Fig. 2), hinting at unknown roles of CWIN in early seed development.

### Shift in Spatial Patterning from Ovule to Seed: *GhCWIN1* Was Highly Expressed in Seed Coat TC Precursors and Syncytial Endosperm

To explore roles of *GhCWIN1* in early seed development, we conducted systematic in situ hybridization experiments on cotton seed from prefertilization to 15 DAA, the onset of synthesis and storage of oils in embryo and cellulose in fiber (Ruan, 2005).

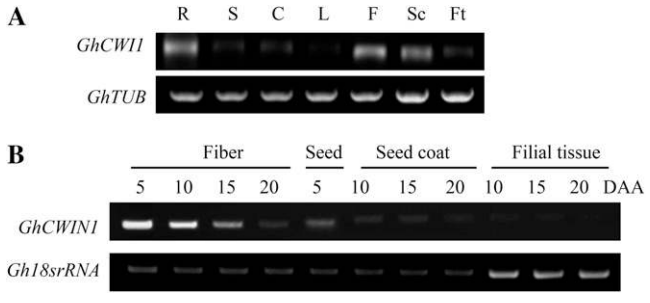


**Figure 1.** Transcript levels of *GhCWIN1* in different cotton tissues (A) and in cotton seeds at different development stages (B). R, Root; S, stem; C, cotyledon; L, source leaf; F, Sc, and Ft represent 10-d fiber, seed coat, and filial tissue, respectively. *GhTUB* and *Gh18srRNA* were used as internal controls for (A) and (B), respectively.

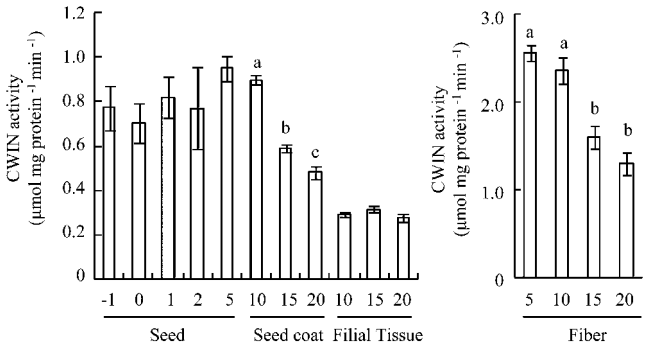


**Figure 2.** CWIN activity in cotton seed, seed coat, filial tissue, and fiber at different development stages. Each value is the mean ± SE of at least three biological replicates. Values with different letters (a, b, and c) indicate significant differences ($P \leq 0.05$) among different stages, whereas values with no letter above the bars indicate no statistically significant differences, according to randomization one-way ANOVA test.

At −2 DAA, in comparison with the sense control (Fig. 3A), *GhCWIN1* mRNA was expressed universally in cotton ovule (Fig. 3B). Interestingly, the spatial distribution of *GhCWIN1* transcripts appeared more restrictive to particular cell layers at 1 DAA (Fig. 3C). They became evident at the innermost layer of the outer seed coat, especially in the chalazal end and at the innermost seed coat bordering the nucellus as well as in the young fibers just initiating from the seed epidermis (Fig. 3C). A close examination highlighted strong *GhCWIN1* signals in the chalazal vein and at the innermost layer of the outer integument (Fig. 3E) in comparison with its sense control (Fig. 3D).

The physiological significance of *GhCWIN1* expression would depend on, in part, the cellular pathway of Suc transport (Ruan et al., 2010). To examine the symplasmic continuity between the two tissues, a phloem-mobile symplastic fluorescent probe, carboxyfluorescein (CF), was ester loaded into shoots through their cut ends for 48 h (Ruan et al., 2001). Based on the positions of the outer and inner seed coat viewed under bright field (Fig. 3F), it is clear that the fluorescent CF signal spread throughout the outer seed coat but was unable to travel into the inner seed coat (Fig. 3G), consistent with previous studies (Ruan et al., 2001). Lack of CF movement in the region was observed even if the feeding time was extended to 72 h. This observation demonstrates a symplasmic discontinuity at the interface between the two tissues, indicating a necessity of the apoplasmic pathway, consistent with the expression of *GhCWIN1* in this cellular site (Fig. 3, C and E).

The transition from ovule to seed is characterized by the coordinated development of endosperm and embryo. The underlying molecular mechanism for the shift, however, remains unknown. Here, we show that *GhCWIN1* may play an important role in generating Glc as signals to stimulate the nuclear division of the syncytial endosperm.

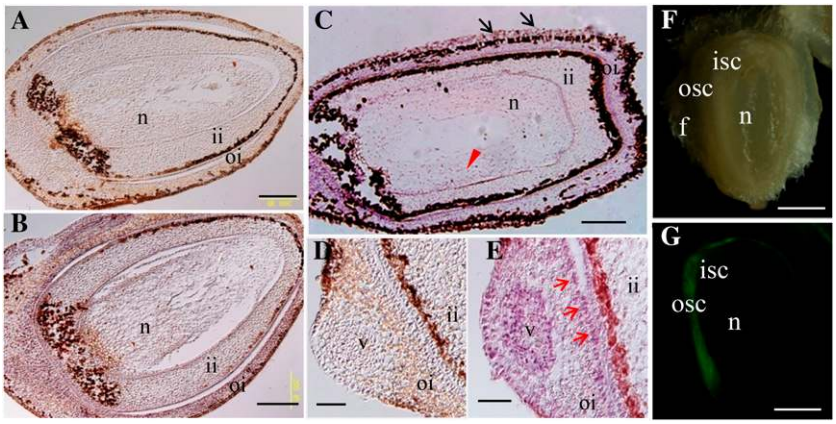


**Figure 3.** Spatial distribution of *GhCWIN1* mRNA in −2-DAA cotton ovule and 1- to 2-DAA cotton seed. A and B, Longitudinal sections of −2 DAA cotton ovule hybridized with sense (A) and antisense (B) RNA probes for *GhCWIN1*. Note the weak and even distribution of *GhCWIN1* mRNA across the section in B. C, A longitudinal section of 1- to 2-DAA cotton seed hybridized with an antisense RNA probe for *GhCWIN1*. Note the *GhCWIN1* mRNA signals in the innermost layer of the inner seed coat (arrowhead) in fibers (arrows). D and E, Magnified views at the main vein of cross-sections of 1- to 2-DAA cotton seed hybridized with sense (D) and antisense (E) probes for *GhCWIN1*. Red arrows in E indicate the strong *GhCWIN1* signals at the innermost layer of the outer integument. Signals were also apparent in chalazal vein (V). F and G, Free-hand sections of 2-DAA cotton seed viewed under bright field (F) and fluorescence microscopy for the symplastic fluorescent dye CF (G). Note that CF signals spread within the outer seed coat but not beyond. f, Fiber; ii, inner integument; isc, inner seed coat; n, nucellus; oi, outer integument; osc, outer seed coat; v, vascular bundle. Bars = 200 $\mu$m (A–C), 50 $\mu$m (D and E), and 500 $\mu$m (F and G).

Apart from its expression in elongating fibers and at the interface between outer and inner seed coat, *GhCWIN1* mRNA was abundantly detected at the innermost layer of the inner seed coat, namely, transfer cell precursors (TCPs; arrows in Fig. 4, B and C, versus a sense control in Fig. 4A) that develop WIs later at 10 DAA, characteristic of TCs (Pugh et al., 2010). Remarkably, *GhCWIN1* transcripts were also localized in embryo sacs at the chalazal end (red arrowhead in Fig. 4B). Developing cotton seed is known to develop nuclear-type endosperm, in which the initial endosperm nucleus divides repeatedly without cell wall formation in early stages, before they become cellularized at approximately 10 DAA (Jensen et al., 1977; Schulz and Jensen, 1977; Ruan et al., 2008). Toluidine blue staining revealed a number of nuclei located at the chalazal end of the embryo sac (Fig. 4D), showing the coenocytic nature of the endosperm in this region at this stage. To confirm the asymmetric distribution of *GhCWIN1* transcripts in the embryo sac, in situ hybridization was further performed on a set of cross-sections cut from chalazal to micropylar ends, as denoted by dotted lines in Figure 4B. These analyses revealed that, within the embryo sac, compared with the sense control (Fig. 4E), *GhCWIN1* transcripts were most abundant near the chalazal end (Fig. 4F), where syncytial endosperm locates (Fig. 4D), and became almost undetectable at the micropylar end (Fig. 4H), and the signals were predominantly detected at the periphery of the embryo sac (Fig. 4, F and G). Accordingly, we conclude that *GhCWIN1* was expressed in syncytial endosperm undergoing nuclear division.

### Shift in Spatial Patterning during Seed Development: Expression of *GhCWIN1* Became Diminished in Seed Coat TCs But Evident in Globular Embryo and in the Provascular Zone of Torpedo Stage Embryo

As the seed develops to 10 DAA, the seed coat TCPs begin to develop intensive WIs, thereby forming functional TCs (Ruan et al., 1997; Pugh et al., 2010). Inside this layer, multicellular proembryo gave rise to globular stage embryo and the endosperm has undergone the cellularization process starting from the micropylar end to the chalazal region (Fig. 5A; Ruan et al., 2008). Coordinating with these developmental changes, *GhCWIN1* exhibited further intrinsic shifts in spatial patterning. One observation was that, in contrast to the abundant *GhCWIN1* mRNAs detected in the TCP of 5-DAA seed (Fig. 4, B, C, and F), the signals of *GhCWIN1* transcripts were reduced sharply at the innermost layer of the inner seed coat at 10 DAA (Fig. 5, C and E, versus their respective sense controls in Fig. 5, B and D), spatially and temporally coinciding with the onset of TC WI formation. Further different from that at 5 DAA (Fig. 4), no *GhCWIN1* transcript signals were found in the cellularizing endosperm at 10 DAA (arrowhead in Fig. 5C). *GhCWIN1* mRNA, however, was expressed in a small area of endospermic parenchyma cells attached to the suspensor at the micropyle end (Fig. 5F). *GhCWIN1* transcript was also detected in the globular embryo (Fig. 5, C and F), as compared with that in the sense control (Fig. 5B).

As development progresses, globular embryo gives rise to the "heart" and then "torpedo" morphologies. By 15 DAA, cotton fiber and seed coat decreased

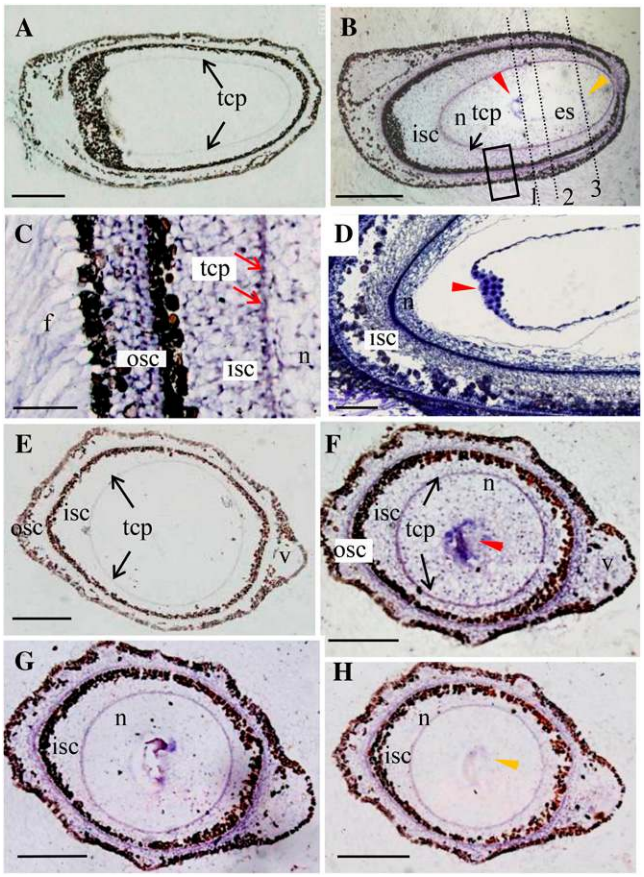


**Figure 4.** Spatial distribution analysis of *GhCWIN1* mRNA in 5-DAA cotton seeds revealed strong signals in TCPs and nuclear endosperm at the chalazal end embryo sac. A and B, Longitudinal sections of 5-DAA cotton seed hybridized with sense (A) and antisense (B) RNA probes for *GhCWIN1*. Note the strong signals in TCP and embryo sac close to the chalazal end, indicated by arrows and the red arrowhead, respectively, in B. The yellow arrowhead indicates the micropylar end of the embryo sac. C, A magnified view of B at the boxed seed coat region. D, A longitudinal section of 5-DAA cotton seed stained with toluidine blue. Note the number of nuclei located at the chalazal end of the embryo sac, as indicated by the red arrowhead, characteristic of the syncytial endosperm at 5 DAA. E to H, Cross-sections of 5-DAA cotton seed hybridized with sense (E) and antisense (F–H) RNA probes for *GhCWIN1*. The relative positions of cross-sections in F to H are denoted in the longitudinal section in B by dotted lines 1 to 3, respectively. Note that *GhCWIN1* mRNAs were most abundant in the nuclear endosperm at the chalazal embryo sac, as indicated by the red arrowhead in F, but became hardly detectable at the micropylar end (H). f, Fiber; isc, inner seed coat; n, nucellus; osc, outer seed coat; tcp, transfer cell precursor; v, vascular bundle. Bars = 500 $\mu$m (A and B), 200 $\mu$m (C), and 50 $\mu$m (C and E–H).

CWIN activity by more than one-third as compared with those at 10 DAA, while CWIN activity remained low in the filial tissue (Fig. 2). Consistently, *GhCWIN1* mRNA signals became hardly detectable in maternal tissues and the embryo-attached micropylar endosperm (Fig. 6, B and D, versus their respective sense controls in Fig. 6, A and C). On the contrary, strong signals of *GhCWIN1* transcripts were apparent within the embryo (Fig. 6D). In the heart-shaped embryo of most plants, the protoderm, procambium, and ground meristem have been formed, which have organized to form the apical meristem, radicle, cotyledons, and hypocotyl in seeds (Ohashi-Ito and Fukuda, 2010). *GhCWIN1* labeling was distributed over all these tissues as compared with the sense control (Fig. 6, D versus C) but was most abundant in a V-shaped region within the torpedo embryo (Fig. 6E), mirroring the zone of provascular development (Tucker et al., 2008).

To assess whether such a development-dependent expression of CWIN is a common feature in other species, we examined in Arabidopsis seeds the expression of *AtCWIN2* (*At3g52600*, *Atßfruct2*) and *AtCWIN4* (*At2g36190*, *Atßfruct5*), the two *CWINs* exclusively expressed in reproductive organs based on microarray data (http://www.weigelworld.org/resources/microarray/AtGenExpress/). Using a gene-specific probe, *AtCWIN2* transcripts were weakly detected in the seed coat and embryo (Fig. 7B) as compared with the sense control (Fig. 7B); *AtCWIN4* labeling was much stronger and distributed over the cells of the seed coat, central vein, embryo, and chalazal endosperm (Fig. 7D), in comparison with the results obtained using sense probe (Fig. 7C). These findings, in conjunction

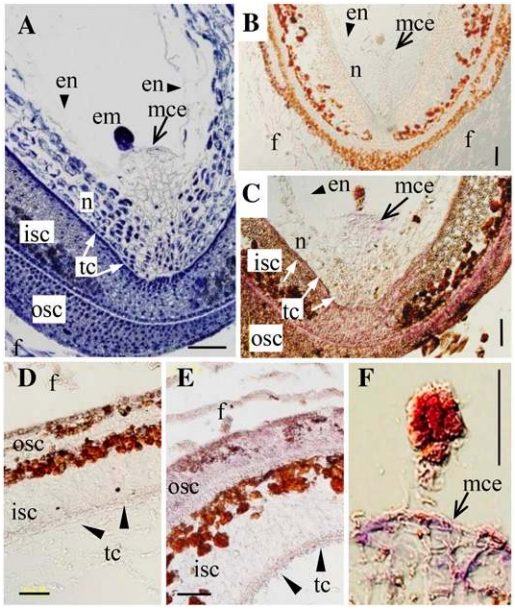


**Figure 5.** Spatial distribution analysis of *GhCWIN1* mRNA in 10-DAA cotton seed. A to C, Longitudinal sections of 10-DAA cotton seed stained with toluidine blue (A) and hybridized with sense (B) and antisense (C) RNA probes for *GhCWIN1*. Arrowheads point to the peripheral endosperm under cellularization that shows no *GhCWIN1* RNA signals, whereas signals were detected in the globular embryo (C versus B and A). D and E, Magnified views of B and C in the seed coat region, respectively, showing little *GhCWIN1* mRNA signals in the TCs. F, A magnified view of C at the micropyle endosperm and embryo. Note the *GhCWIN1* mRNA signals detected in the globular embryo and in the underlying two to three layers of cellularized endosperm cells. em, Embryo; en, endosperm; f, fiber; isc, inner seed coat; mce, micropylar cellularizing endosperm; n, nucellus; osc, outer seed coat; tc, transfer cell. Bars = 100 $\mu$m (A and B) and 50 $\mu$m (D–F).

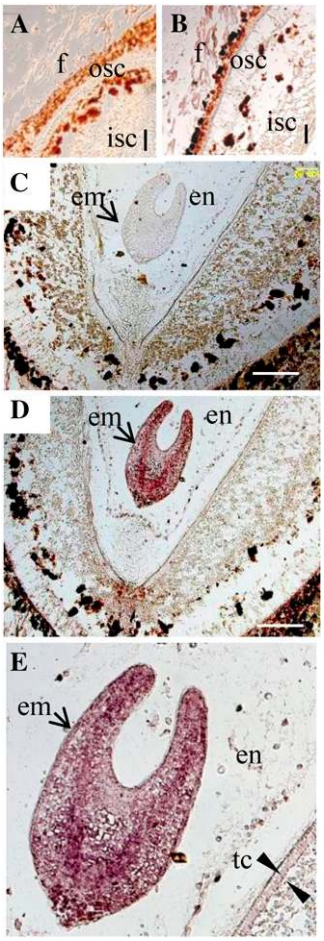


**Figure 6.** Spatial distribution analysis of *GhCWIN1* mRNA in 15-DAA cotton seed. A and B, Magnified views of longitudinal sections at integument hybridized with sense (A) and antisense (B) RNA probes for *GhCWIN1*. C and D, Longitudinal sections at micropylar ends hybridized with sense (C) and antisense (D) RNA probes for *GhCWIN1*, showing strong signals in the central region of the torpedo embryo. E, Magnified view of D, showing a strong V-shaped signal pattern of *GhCWIN1* within the torpedo embryo. em, Embryo; en, endosperm; f, fiber; isc, inner seed coat; osc, outer seed coat; tc, transfer cell. Bars = 50 $\mu$m (A, B, and E) and 200 $\mu$m (C and D).

with the microarray data, suggest that *AtCWIN4* is a major CWIN gene expressed in Arabidopsis seed, similar to the scenario for *GhCWIN1* in cotton seed. Interestingly, *AtCWIN4* mRNA was abundant in chalazal endosperm (red arrowhead in Fig. 7, D versus C.) undergoing endoreduplication but much weaker in cellularizing micropylar endosperm corresponding to the heart-embryo stage (Boisnard-Lorig et al., 2001), consistent with *GhCWIN1* transcripts being detected in cotton chalazal endosperm undergoing nuclear division (Fig. 4F) but not in cellularizing endosperm (Fig. 5). Within the embryo, strong *AtCWIN4* expression was detected from globular stage to torpedo stage (Fig. 7, E–G). Similar to *GhCWIN1* mRNA in cotton embryo, *AtCWIN4* transcripts were present in all cells of the globular embryo proper (Fig. 7E) and early heart stage embryo (Fig. 7F). The transcript signals became more restricted to the centers of embryonic organs, forming a Y shape in torpedo stage embryo (Fig. 7G). Toluidine blue staining showed a file of elongated cells along the midlines of the cotyledons as well as of the embryo axis (Fig. 7H), which was identified previously as provascular cells (Baima et al., 1995; Hardtke and Berleth, 1998; Tucker et al., 2008). Thus, *AtCWIN4* probably is highly expressed in the provascular tissues during embryogenesis.

## DISCUSSION

Seeds originate from ovules, starting with the development of maternal tissues, followed by the coordinated growth and differentiation of embryo and endosperm upon double fertilization. Extensive studies have shown essential roles of CWIN in seed filling, probably through regulating Suc unloading and the establishment and maintenance of sink strength (Cheng et al., 1996; Weber et al., 1996; Wang et al., 2008; Jin et al., 2009; Zanor et al., 2009). However, it remains largely unknown how CWIN exerts its regulation early in seed development (Ruan et al., 2010). Here, by systematically examining the spatial and temporal expression of *GhCWIN1* in cotton seed early in development, we uncovered several novel observations that suggest new roles of GhCWIN1 in TC differentiation, endosperm nuclear division, and embryonic provascular development.

### GhCWIN1 May Negatively Regulate TC Differentiation

TCs are specialized cells exhibiting invaginated WIs. In cotton seed, TCs begin to form WIs at approximately 10 DAA (Pugh et al., 2010). The findings that *GhCWIN1* was strongly expressed in TCPs in this cell layer at 1 to 5 DAA (Figs. 3 and 4) but was reduced to an almost undetectable level at 10 DAA onward (Figs. 5 and 6) suggest a negative role of CWIN in TC differentiation. Consistent with this view is a recent report showing that Glc functions as a negative signal for TC WI induction in faba bean cotyledons (Andriunas et al., 2011). The high expression of *GhCWIN1* in TCP at 1 to 5 DAA likely generates high amounts of Glc, which could help to hold these cells in an undifferentiated state. On the other hand, the sharp decrease of *GhCWIN1* transcript level at 10 DAA probably terminates the inhibition, allowing TC differentiation to proceed. In agreement with this proposition, a progressive reduction of CWIN activity was also detected in faba bean cotyledon development, which temporally and spatially coincided with the onset of TC differentiation in abaxial epidermal cells (Weber et al., 1996, 1997; Offler et al., 1997). Significantly, in contrast to the absence of *GhCWIN1* expression in the TCs during WI formation, *Sus* was highly expressed in TCs at 10 DAA onward and its abundance was strongly correlated with the degree of TC WIs in cotton seed (Pugh et al., 2010). Their contrasting temporal expression patterns indicate distinctive roles of CWIN and *Sus* in TC development, with the former acting as a

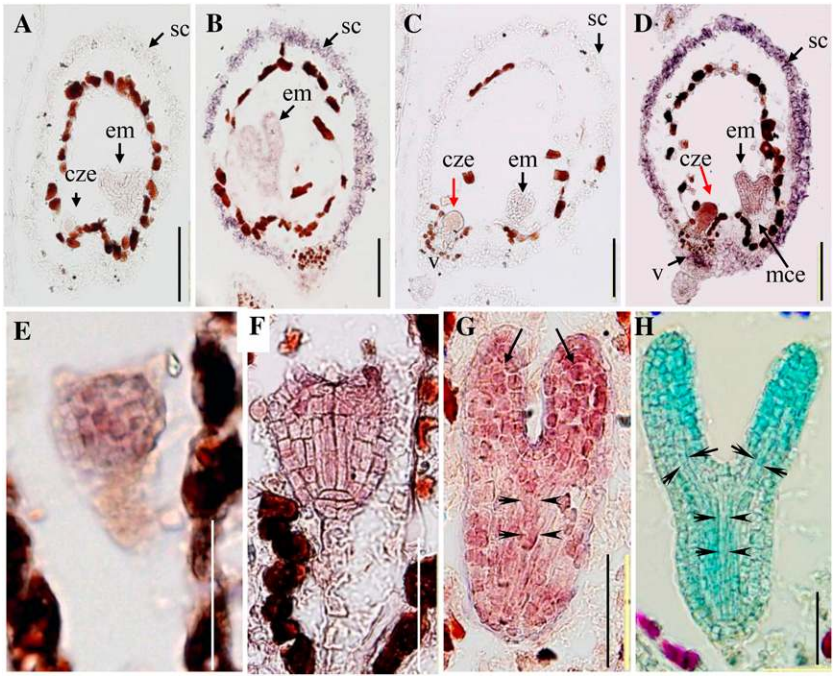


**Figure 7.** Spatial distribution analysis of *AtCWIN2* and *AtCWIN4* transcripts in Arabidopsis seeds. A and B, Longitudinal sections of heart-embryo stage Arabidopsis seed hybridized with sense (A) and antisense (B) RNA probes for *AtCWIN2*, showing weak signals in embryo and seed coat. C and D, Longitudinal sections of heart-embryo stage Arabidopsis seed hybridized with sense (C) and antisense (D) RNA probes for *AtCWIN4*, showing strong signals in chalazal endosperm, embryo, and seed coat. Note that no or little *AtCWIN4* mRNAs were detected in the micropylar end endosperm that has undergone cellularization at this stage. E to G, A series of longitudinal sections of Arabidopsis embryos at global stage (E), early heart stage (F), and torpedo stage (G) hybridized with *AtCWIN4* antisense probe. H, A longitudinal section of torpedo stage embryo stained with toluidine blue, showing the provascular region in the middle, highlighted by arrowheads. cot, Cotyledon; cze, chalazal end endosperm; em, embryo; mce, micropylar end endosperm; sc, seed coat; v, vascular bundle. Bars = 100 $\mu$m (A–D) and 50 $\mu$m (E–H).

repressor for TC differentiation and the latter being a positive regulator for TC WIs.

### GhCWIN1 Is Involved in Endosperm Nuclear Division But Not in Cellularization

Another important observation of this study is the abundant *GhCWIN1* mRNA detected in syncytial endosperm in the embryo sac near the chalazal end (Fig. 4, B, E, and F; see "Results"). The signal, however, was not detectable at the later stages (Figs. 5, B and C, and 6D) when the endosperm is undergoing cellularization (Ruan et al., 2008). Similar results were observed in Arabidopsis, where *AtCWIN4* was also expressed strongly in syncytial endosperm at the chalazal end but not in cellularizing micropylar endosperm (Fig. 7D). These findings are of significance for three reasons.

First, despite endosperm being central to seed development, there are few studies on the temporal expression of genes in the tissue, spanning from nuclear division to cellularization, thus hampering our understanding of the regulation of seed development. To our knowledge, our finding represents the first report on the expression of *CWIN* in nuclear endosperm.

Second, our findings here indicate that CWIN could promote nuclear division in early endosperm development, probably through sugar signaling. By cross talking with hormones, invertase-mediated sugar signaling may regulate the expression of cell cycle control genes, thereby modulating chromosomal duplication and cell cycles (Hartig and Beck, 2006), as proposed for embryonic cell division in legumes (Weber et al., 2005) and maize (Rijavec et al., 2009; LeClere et al., 2010). Consistent with this is the strong expression of *GhCWIN1* and *AtCWIN4* transcripts in globular embryos of cotton (Fig. 5F) and Arabidopsis (Fig. 7E), respectively, that are undergoing rapid cell division. Importantly, genetic evidence shows that hexoses released from CWIN activity, rather than from exogenous supply, are critical for mitotic division (Cheng and Chourey, 1999). Stimulation of endosperm cell division by hexose has been observed in the monocotyledonous maize (Cheng et al., 1996; Vilhar et al., 2002). However, in maize and barley grains, CWIN is specifically localized in basal endosperm TCs at the prestorage to storage phase (Cheng et al., 1996; Dumas and Rogowsky, 2008), and it remains to be determined if CWIN is expressed in maize nuclear endosperm.

Third, the absence of *GhCWIN1* and *AtCWIN4* expression in cellularizing endosperm shows that CWIN is not responsible for the cell wall biosynthesis of endosperm. Interestingly, a recent work in Arabidopsis revealed a failure of endosperm cellularization correlated with increased hexose content in the seed (Hehenberger et al., 2012), suggesting that a low hexose level is required for endosperm cellularization. Indeed, compared with 5-DAA cotton seed, Glc and Fru content significantly declined in 10-DAA filial tissues (Supplemental Fig. S4), possibly contributed, in part, by the down-regulation of GhCWIN1 expression in endosperm from the syncytial to the cellularizing stage (Figs. 4 and 5). In contrast to GhCWIN1, GhSus is barely detectable in 5-DAA cotton seed, except for the young fiber cells (Ruan et al., 2003; Ruan, 2005) but is highly expressed in cellularizing endosperm of cotton seed (Ruan et al., 2008). Suppression of *Sus* reduces cellulose and callose biosynthesis, thereby disrupting cell wall integrity (Ruan et al., 2008). It is thus clear that CWIN and *Sus* appear to play distinctive roles in endosperm development, with the former generating Glc signals for nuclear division and the latter being

responsible for cell wall biosynthesis and, hence, the cellularization of endosperm.

### Roles of GhCWIN1 in Embryonic Vascular Development

It is remarkable that as embryogenesis progressed, *GhCWIN1* expression became more zone specific within the torpedo embryo, exhibiting a V-shaped pattern (Fig. 6E). Analysis in Arabidopsis also revealed a similar Y-shaped pattern of *AtCWIN4* expression in torpedo embryo (Fig. 7G). Based on their elongated morphology and central position in cotyledon and radicel, they are most likely provascular tissues. This observation raises a fascinating question on the role of CWIN in provascular development. One possible mechanism may relate to cross talk between auxin and sugar signaling conditioned by CWIN (Ruan et al., 2012). During embryogenesis, procambial cells undergo coordinated, asymmetric cell divisions, giving rise to vascular precursor cells. In Arabidopsis, vascular pattern formation in early embryo depends on auxin biosynthesis, transport, and signaling (for review, see Berleth et al., 2000; De Smet and Jürgens, 2007; Möller and Weijers, 2009). Interestingly, recent work on the maize CWIN-deficient *miniature1* mutant revealed that a decreased hexose-to-Suc ratio in basal kernel resulted in a reduction in Trp-dependent indole-3-acetic acid biosynthesis, due to down-regulation of a *YUC* gene encoding a rate-limiting enzyme flavin monooxygenase, YUCCA (Chourey et al., 2010; LeClere et al., 2010). These observations suggest that CWIN may regulate provascular development by modulating auxin biosynthesis via sugar signaling.

### Models of CWIN- and Sus-Mediated Early Seed Development

The cellular domain- and stage-specific expression of CWIN and *Sus* as discussed above allows us to propose models of CWIN- and Sus-mediated early seed development as summarized in Figure 8 and discussed below.

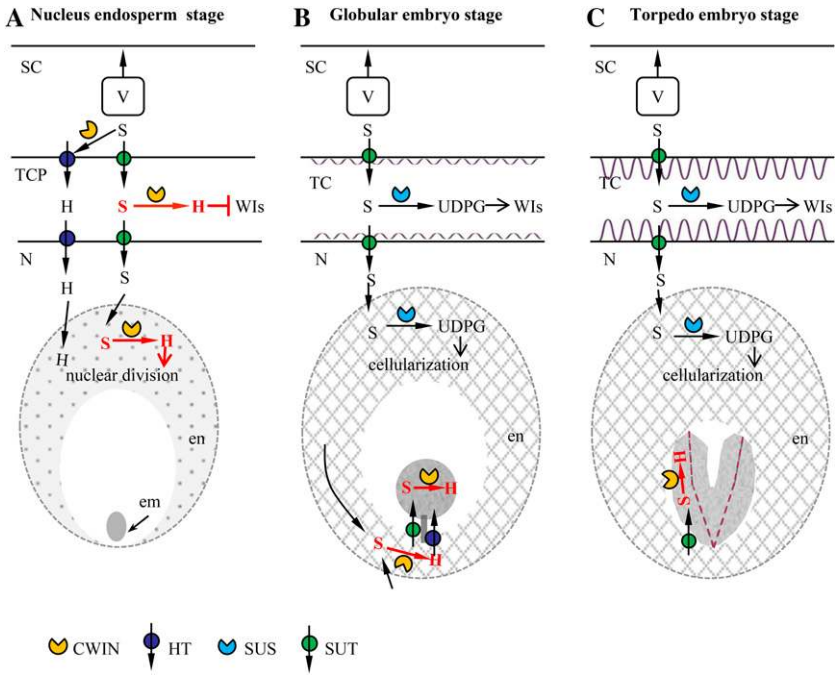


**Figure 8.** Models of CWIN- and *Sus*-mediated early seed development at the stages of nucleus endosperm (A), globular embryo (B), and torpedo embryo (C). A, After phloem unloading in the seed coat, part of the Suc is partitioned outward to the developing fibers (data not shown), while the remainder is hydrolyzed by GhCWIN1 at the interface between the outer and inner seed coat and at the TCPs located at the innermost layer of the seed coat. The resultant hexose serves as a signal to (1) repress TC development and (2) stimulate endosperm nuclear division after being transported by putative hexose transporters (Supplemental Fig S3). Suc also flows to the embryo sac, possibly mediated by Suc transporters (Supplemental Fig S3; Ruan et al., 2001), where it is hydrolyzed by CWIN within the syncytial endosperm for nuclear division. B, At the globular embryo stage, *GhCWIN1* expression became undetectable in the seed coat. Suc is degraded by *Sus* at the innermost layer of the seed coat for TC WIs (Ruan et al., 1997; Pugh et al., 2010) or moved toward the filial tissues mediated by Suc transporters (Ruan et al., 2001). Suc is cleaved by *Sus* in the cellularizing endosperm for cell wall biosynthesis (cellularization; Ruan et al., 2008). Some Suc is transported to the globular embryo for CWIN-mediated hydrolysis, yielding Glc for cell division. Involvement of Suc and hexose transporters in delivering sugars to the globular embryo is indicated by the expression of these genes in the filial tissue (Supplemental Fig S3). C, Model of CWIN- and *Sus*-mediated seed development at the torpedo stage is similar to that at the globular embryo stage, except for the stronger expression of *Sus* in the TC for intensive WIs (Pugh et al., 2010) and of GhCWIN1 in the central region of the torpedo embryo for provascular development. em, Embryo; en, endosperm; H, hexoses; N; nucellus; S, Suc; SC, seed coat; V, vascular bundle; WIs, wall ingrowths.

After double fertilization, the endosperm undergoes nuclear division earlier and faster than the embryo (Berger, 1999; Bate et al., 2004). In cotton, nuclear division of endosperm starts immediately after fertilization, while the fertilized egg does not divide until several days later (Stewart, 1986). Maintaining such a developmental sequence is important for proper seed development (Ruan et al., 2008). However, the underlying mechanism is unknown. Here, we propose that strong expression of *GhCWIN1* in syncytial endosperm but not in the embryo sac where the fertilized egg sits (Figs. 4 and 8A) provides a basis for generating Glc as signals to stimulate endosperm nuclear division but not embryonic cell division. Consistent with this hypothesis is the localization of a CWIN inhibitor at the boundary between the endosperm and embryo in 5-d maize seed (Bate et al., 2004), which could help to minimize Glc flow to the young embryo at this stage. CWIN in the TCP at the innermost area of the seed coat (Fig. 8A) may also provide hexoses to liquid endosperm for nuclear division in addition to its potential role in repressing TC WIs, as discussed previously.

As cotton seed developed to the globular embryo stage, *GhCWIN1* transcripts became undetectable in TC and cellularizing endosperm but were evident in the embryo (Fig. 8B). At this stage, Suc is degraded by *Sus* in the TCs and in cellularizing endosperm for cell wall biosynthesis (Ruan et al., 2008; Pugh et al., 2010). Some Suc must also flow to the globular embryo, probably via the micropylar endosperm, as indicated by studies in Arabidopsis and oilseed rape (*Brassica napus*) seeds (Baud et al., 2005; Morley-Smith et al., 2008), where they may be hydrolyzed by GhCWIN1 into hexoses in the embryo for cell division (Fig. 8B). It is intriguing why *GhCWIN1* transcripts were also present in some endosperm cells connecting the suspensor at the micropylar end (Fig. 5F). One possibility is that it may facilitate Suc flow toward the young embryo by lowering Suc concentration at the basal suspensor (Fig. 8B). In agreement with this, the expression of the cotton Suc transporter gene *GhSUT1* (GenBank accession no. EF453641) as well as a monosaccharide transporter gene, *GhMST1* (GenBank accession no. AI731272), the only Suc and hexose transporter genes identified from cotton fiber and seed EST databases, was increased significantly in 10-DAA filial tissue as compared with those in 5-DAA seed (Supplemental Fig. S3), implying the importance of these transporters in sugar delivery for endosperm cellularization and embryo development. Evidence is also accumulated from Arabidopsis that a Suc transporter gene, *AtSUT5*, is specifically and highly expressed in micropylar endosperm cells adjacent to the embryo during early stages of seed development (up to the torpedo stage) and is necessary for normal rates of embryo development (Baud et al., 2005).

The overall model of carbon flow in cotton seed in the torpedo-embryo stage (Fig. 8C) is similar to that in the globular-embryo stage based on expression patterns of *GhCWIN1* (Figs. 5 and 6) and *Sus* (Ruan et al., 2003, 2008; Pugh et al., 2010). However, the torpedo embryo may receive much of its carbon in the form of Suc rather than hexose, suggested by the enhanced expression of *GhSUT1* but slightly reduced expression of *GhMST1*, respectively (Supplemental Fig. S3). The major difference between the two stages is that *CWIN* is expressed all over the globular embryo (Figs. 5F and 7E) but mainly in the central zone of the torpedo-stage embryo (Figs. 6E and 7G), possibly regulating provascular development in the latter stage.

In conclusion, this study presents novel data on the cellular domain- and developmental stage-specific expression of *GhCWIN1* in cotton seed early in development, which provides new insights into its possible roles in TC differentiation, endosperm nuclear division, and embryonic provascular development. By comparing with previous studies of *Sus* expression in cotton seed in conjunction with those of Suc and hexose transporters, we propose that CWIN mainly plays regulatory roles in TC and filial tissue development through sugar signaling, whereas *Sus* is largely involved in cell wall biosynthesis. Further studies are required to gain in-depth understanding of the mechanisms by which CWINs regulate early seed development by using, for example, high-resolution imaging and measurement of sugars and other related metabolites at the single-cell level across different stages of seed development.

## MATERIALS AND METHODS

### Plant Materials

Cotton (*Gossypium hirsutum* 'Coker 315') plants were grown in a glasshouse at 28°C with a photoperiod of 14 h of light and 10 h of dark. Cotton fruit age was determined by tagging each pedicel on the day of anthesis. Arabidopsis (*Arabidopsis thaliana* ecotype Columbia-0) seeds were surface sterilized and germinated on one-half-strength Murashige and Skoog medium containing 1% Suc. Plates were incubated at 4°C in darkness for 2 to 3 d and then moved to a growth chamber at 22°C with a photoperiod of 12 h of light and 12 h of dark. After 1 week, seedlings were transferred to soil and were grown in a chamber at 22°C with a photoperiod of 16 h of light and 8 h of dark. For RNA extraction and enzyme assay, samples were frozen in liquid $N_2$ and stored at −70°C until analysis. For in situ hybridization, fresh cotton ovules/seeds were collected and fixed immediately in 4% (w/v) paraformaldehyde, dehydrated in ethanol, and embedded in paraffin using our established protocol (Jin et al., 2009).

### Cloning and Sequence Analysis of *GhCWIN1*

Total RNA was isolated from five cotton seeds and other specified tissues according to Ruan et al. (1997). First-strand cDNA was synthesized using the SMART RACE cDNA Amplification Kit (Clontech), following the manufacturer's instructions. A 633-bp cotton EST sequence (GenBank accession no. AI725433) was identified as a cDNA fragment of the cotton CWIN gene by BLASTP analysis. According to this EST sequence, gene-specific primers for 5′ and 3′ RACE were designed and are listed in Supplemental Table S2, along with the thermal cycling conditions. PCR products were separated by electrophoresis on a 1.0% agarose gel. The purified PCR products were cloned into pGEM T-Easy Vector (Promega) and then sequenced using the 3730 XL DNA analyzer (Applied Biosystems).

The DNA Star software package (version 5.02) was used to assemble the cDNA fragment sequence and to find the ORF of full-length cDNA. Sequence comparison and phylogenetic analysis were performed by MEGA version 4

software (Tamura et al., 2007). Sequences were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The tree was constructed by the unweighted pair group method with arithmetic mean, with statistical analysis by the bootstrap method using 1,000 repetitions. The sequences used for the phylogenetic analyses are listed in Supplemental Table S1.

## Semiquantitative RT-PCR Analyses

One microgram of RNA was treated by RQ1 DNase (Promega), and the DNA-free RNA was reverse transcribed to cDNA using oligo(dT) primer with Moloney murine leukemia virus reverse transcriptase (Toyobo) following the manufacturer's instructions. A specific 359-bp fragment of *GhCWIN1* was amplified using GhCWIN1-RT primers. GhINH1-RT primers were used to generate a specific 315-bp fragment of the probable cotton CWIN inhibitor gene *GhINH1*. Gene-specific primers were designed to amplify 378- and 194-bp fragments of cotton monosaccharide transporter *GhMST1* and Suc transporter *GhSUT1*, respectively. For internal controls, a 500-bp cotton 18S rRNA gene fragment and a 308-bp *GhTUB2* fragment were amplified with Gh18srRNA-RT primers and GhTUB-RT primers, respectively. By adjusting the parameters according to the gene expression levels in different templates, all the RT-PCR-amplified products presented in this study were in linear ranges. The primer sequences and PCR parameters are listed in Supplemental Table S2.

## In Situ Hybridization

In situ hybridization was carried out using our established protocol (Jin et al., 2009). To generate gene-specific probes of *GhCWIN1*, a 346-bp fragment, ranging from 1,473 bp downstream of the start codon to 51 bp downstream of the stop codon (3′ untranslated region), was amplified using GhCW1-in situ primers. Gene-specific 677-bp *AtCWIN2* fragment and 718-bp *AtCWIN4* fragment were amplified using the two primer sets of AtCW2-in situ and AtCW4-in situ, respectively. All primer sequences and PCR conditions are listed in Supplemental Table S2. Fragments were cloned into pBluescript II SK+/− and linearized with *Bam*HI and *Xba*I for the generation of sense and antisense probes, respectively. Sense and antisense RNA transcripts were synthesized by T3 and T7 RNA polymerase with digoxigenin-UTP (Roche Diagnostics) as the label.

## Invertase Enzyme Assay and Sugar Measurement

Invertase activity and sugar levels were measured enzymatically as described by Wang et al. (2010). Each biological replicate consisted of three technical replicates. Experiments were repeated at least three times, and similar results were obtained. One representative set of data was used for analyses as described in this paper.

## Statistical Analysis

Data were analyzed by randomization one-way ANOVA for analyses of CWIN activity and different sugar contents in cotton seed, seed coat, and filial tissue across multiple time points during seed development. Means were compared using LSD tests in all analyses mentioned above. All statistical calculations were performed using JMP 9 statistics software.

## Supplemental Data

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

**Supplemental Figure S1.** Alignment of the *GhCWIN1* sequence with five plant CWINs (*Atßfruct2*, *Lin6*, *NtCWIN1*, *OsCWIN1*, and *ZmCWIN1*) and two VINs (*Atßfruct4* and *GhVIN1*).

**Supplemental Figure S2.** Phylogenetic analysis classifies GhCWIN1 as a plant CWIN.

**Supplemental Figure S3.** Transcript levels of cotton CWIN *GhCWIN1*, invertase inhibitor *GhINH1*, monosaccharide transporter *GhMST1*, and Suc transporter *GhSUT1* in cotton seed and filial tissue at different development stages.

**Supplemental Figure S4.** Glc, Fru, and Suc levels in cotton seed, seed coat, and filial tissue during development.

**Supplemental Table S1.** GenBank accession numbers of invertase genes used in Supplemental Figure S2.

**Supplemental Table S2.** Primers and PCR conditions used in this study.

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