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Evidence that High Activity of Vacuolar Invertase Is Required for Cotton Fiber and Arabidopsis Root Elongation through Osmotic Dependent and Independent Pathway,

Respectively

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Abstract

Vacuolar invertase (VIN) has long been considered as a major player in cell expansion. However, direct evidence for this view is lacking due to, in part, the complexity of multi-cellular plant tissues. Here, we used cotton fibers, fast growing single-celled seed trichomes, to address this issue. VIN activity in elongating fibers was ~4-6-fold higher than that in leaves, stems and roots. It was undetectable in fiberless cotton seed epidermis but became evident in initiating fibers and remained high during their fast elongation and dropped when elongation slowed. Further, genotype with faster fiber elongation had significantly higher fiber VIN activity and hexose levels than that slow-elongating genotype. By contrast, cell wall or cytoplasmic invertase activities did not show correlation with fiber elongation. To unravel the molecular basis of VIN-mediated fiber elongation, we cloned GhVIN1 that displayed VIN sequence features and localized to vacuole. Once introduced to Arabidopsis, GhVIN1 complemented the short root phenotype of a VIN T-DNA mutant and enhanced the elongation of root cells in the wild-type. This demonstrates that GhVIN1 functions as VIN in vivo. In cotton fiber GhVIN1 expression level matched closely with VIN activity and fiber elongation rate. Indeed, transformation of cotton fiber with GhVIN1 RNAi or over-expression constructs reduced or enhanced fiber elongation, respectively. Together, the analyses provide a set of evidence on the role of VIN in cotton fiber elongation mediated by GhVIN1. Based on the relative contribution of sugars to sap osmolality in cotton fiber and Arabidopsis root, we conclude that VIN regulates their elongation in an osmotic dependent and independent manner, respectively.
Introduction

Sucrose is the principal end product of photosynthesis in higher plants and the major carbohydrate translocated from source to sink tissues through phloem. Sucrose cleavage, serving as a starting point for various carbohydrate metabolic pathways, is catalyzed by sucrose synthase (EC 2.4.1.13, Sus) and invertase (β-fructofuranosidase; EC 3.2.1.26). In contrast to the reversible reaction of Sus, invertase irreversibly hydrolyzes sucrose to fructose and glucose. This hydrolysis step is required for the development of many sink tissues and their responses to various stresses (Sturm, 1999; Weschke et al., 2003; Roitsch and González, 2004; Huang et al., 2007; Essmann et al., 2008; Jin et al., 2009; see Ruan et al., 2010 for a recent review).

Based on their pH optimums and subcellular localizations, invertases are classified into three isoforms: a nonglycosylated cytosolic invertase (CIN), with an optimal pH of 7.0-7.8 and highly glycosylated acid invertases with an optimum pH of 3.5 ~ 5.5 either tightly bound to cell wall (CWIN) or appeared as soluble form inside vacuole (VIN) (Roitsch and González, 2004).

Mutational and transgenic studies have established the critical roles of CWIN in the development of seed (Cheng et al., 1996; Ruan et al., 2003), pollen (Roitsch et al., 2003), root (Tang et al., 1999) and leaf and fruit (Jin et al., 2009). By contrast, much less is known about the function of VIN or CIN (Ruan et al., 2010).

High VIN expression or activity has been observed in a range of expanding tissues including maize ovaries (Andersen et al., 2002; McLaughlin and Boyer, 2004), grape berry (Davies and Robinson, 1996), carrot taproot (Tang et al., 1999) and sugar beet petioles (González et al., 2005). It is hypothesized that VIN may play a major role in plant cell expansion, a key step in plant cell development (e.g. González et al., 2005). However, progress in determining roles of VIN in cell expansion suffers from several experimental limitations. Most notably, the multi-cellular nature of
plant tissues makes it difficult to quantitatively evaluate the contribution of VIN in specific cell
types. For example, decrease of VIN expression is associated with maize ovary abortion or
reduction in its expansion (Andersen et al., 2002; McLaughlin and Boyer, 2004). The VIN gene,
Ivr2, however, is expressed in nucellus and vascular bundles of the pedicel deeply embedded within
the pericarp (Andersen et al., 2002). This inherent anatomical feature makes it challenging to
experimentally assess the role of invertase (INV) in these cells.

In this context, developing cotton fiber offers a tractable experimental system to study the role of
INV in cell expansion for the following reasons. First, after initiation from ovule epidermis at
anthesis, the single-celled cotton fibers undergo rapid and synchronized unidirectional expansion to
several centimeters long by ~ 18 d after anthesis (DAA) (Ruan et al., 2001). Hence, a large quantity
of homogenous single cells can be readily harvested for studying the control of cell expansion
(Ruan, 2007). Second, compelling evidence has indicated a major role of osmotically active solutes
in fiber elongation through generation of cell turgor (Ruan et al., 2004). To this end, sucrose moves
into fibers symplasmically early in elongation (Ruan et al., 2001) and hexoses accumulated in the
vacuole are major osmotically active solutes in the fiber sap (Dhindsa et al., 1975; Ruan et al., 1997)
where VIN activity has been reported (Wäfler and Meier, 1994). These observations raise the
possibility that VIN may be a central player in osmotically-driven fiber expansion (Andersen et al.,
2002; Ruan, 2005). Finally, elucidating the role of VIN in cotton fiber could help us not only better
understand the control of rapid cell expansion, but may also identify novel ways to increase fiber
length, a key quality and yield determinant of cotton, the most important textile crop worldwide
(Ruan, 2005).

This study aims to examine the role of VIN in cell expansion by using cotton fiber as a model,
coupled with integrative analyses on elongating root of Arabidopsis. A combination of cellular,
biochemical and molecular genetic analyses show that (i) rapid fiber expansion requires high activity of VIN which is probably exerted by expression of GhVIN1 and (ii) impact on cotton fiber and Arabidopsis root elongation by VIN is most likely achieved through an osmotic dependent and independent manner, respectively.

Results

Vacuolar, but not cell wall or cytoplasmic, invertase activity correlates with cotton fiber elongation developmentally and genotypically

Initial experiments were conducted to compare invertase activities in developing fibers with other tissues. Figure 1A shows that the VIN activities was about 4~6 fold higher in rapidly elongating fibers than that in other tissues including roots, stems and leaves. By contrast, the activities of CWIN and CIN were only about 40% and 8% of VIN, respectively, in 10 d fiber (Fig 1A). The findings imply a role of VIN in fiber elongation.

We next examined if temporal changes of VIN activity correlates with timing and magnitude of fiber elongation during development. To this end, cotton fiber elongation initiates on day of anthesis (Ruan, 2007) where it is technically difficult to dissect fiber initials from the seed coat for enzyme assay. Thus, invertase activity was assayed histologically on whole ovule or seed at this early stage. The analysis revealed strong invertase activities in the fiber-enriched seed surface at 0 and 1 day after anthesis (DAA) but not at -1 DAA prior to fiber initiation (Fig 1B). Measurement of whole seed extracts indicates that the invertase activity detected from the histological assay reflects mainly the VIN but not the CWIN or CIN activity. This is because the VIN activity increased by 70% from -1 to 0 and 1 DAA (Fig 1C) which correlates with what observed from the histological assay (Fig 1B). By contrast, CWIN and CIN activities were less than 50% and 10% of the VIN at 0 and 1
DAA and remained unchanged from -1 to 1 DAA (Fig 1C).

To gain genetic evidence that VIN activity is required for the onset of fiber elongation, a fiberless seed mutant, Xu-142 fuzzless-lintless (Yu *et al.*, 2000) was compared with its wild-type background Xu-142. The histological assay detected no or little invertase activity in the 0- and 1-d seed epidermis of the fiberless mutant (Supplemental Fig 1B), nor in the -1 d wild-type ovule epidermis where fibers have not initiated (Supplemental Fig 1A). As a positive control, strong invertase signals were detected in the wild-type seed at 0 and 1 DAA (Supplemental Fig 1A). Consistently, activity assay on whole seed extract showed ~35% increase in VIN activity from -1 to 0 and 1 DAA in the wild-type Xu-142. By contrast, no such an increase was observed in the extract from the mutant seed (Supplemental Fig 1C).

Fibers at 5 DAA onwards are long enough for free-hand harvesting (Ruan, 2007). Thus, enzyme assay was further performed on fibers over their entire elongation period. Figure 1D shows that VIN activity was 2-3 times higher in 5- and 10- d fibers than that at 15-20 DAA, representing fast and slow elongation phase, respectively (Ruan *et al.*, 2001). By comparison, CWIN and CIN activities were much lower and remained virtually constant throughout fiber elongation (Fig 1D).

To examine further as whether high VIN activity is required for rapid fiber cell elongation as suggested from data described above, another genotype, *Gossypium barbadense, Gb*, with longer fibers than *Gh* (see Ruan *et al.*, 2004) was chosen for comparative studies. Figure 2A shows significantly higher VIN activity in *Gb* than that of *Gh* in fibers throughout the elongation phase, especially at 10 and 15 DAA when the VIN activity in *Gb* was more than doubled as compared to that in *Gh*. By contrast, CWIN activity was less than half of the VIN and showed only a small difference between the two genotypes during fiber elongation (Fig 2A). CIN activity was less than 10% of VIN and remained virtually unchanged during fiber elongation (Data not shown).
Importantly, in comparison with that of the short fiber genotype *Gh*, the higher VIN activity in *Gb* (Fig 2A) corresponds to 50-60% increase in fiber glucose and fructose concentrations and 35% increase in sucrose levels (Fig 2B) at 10 DAA. The hexose levels were about halved at the slow elongation phase of 15 DAA (Figs 2B). Noteworthy is that glucose and fructose were about equal in concentration whereas sucrose was less than 10% of the hexoses (Fig 2B), consistent with an active role of VIN in elongating fiber. The high VIN activity and hexose level in *Gb* corresponded increased fiber length at 10 DAA onwards as compared to that of *Gh* (Fig 2C). However, the faster elongation rate in *Gb*, as indicated by a higher slope, appeared from 5 DAA onwards (Fig 2C).

**Cloning of GhVIN1 that localizes to vacuole**

The strong developmental and genotypic correlation between VIN activity and fiber elongation (Figs 1 and 2) inspired us to explore the molecular basis of VIN activity in cotton fiber. To achieve this, we screened a cotton cDNA library prepared from fiber and ovule and identified a full length clone, designated *GhVIN1*. TBLASTN and BLASTP searches showed a much higher similarity of *GhVIN1* with plant VINs than that of CWINs. For example, *GhVIN1* shared 70%, 68% and 66% amino acid identities with VIN from *Lagenaria siceraria*, *Pyrus pyrifolia* and *Prunus cerasus* (Genbank accession number of AF519809, BAG30919 and AAL05427, respectively) but only 37% with CWIN from *Nicotiana tabacum* and *Lycopersicon esculentum* (Genbank accession number of X81834 and AY634311, respectively).

Further bioinformatics analyses revealed that *GhVIN1* exhibited thirteen regions conserved in known acid invertases, including the characteristic $\beta$-fructosidase motif (NDPD/NG) and cysteine catalytic domain (WECP/VD) (see Ji *et al.*, 2005). Importantly, *GhVIN1* exhibited distinct features of VINs but not CWINs. Here, the N-terminus of *GhVIN1*, as that in other three VINs of LeVIN1,
OsINV3 and ZmIvr1, had a site of “LL/ PLP”, followed by a strong basic region, a hydrophobic transmembrane segment, and a conserved motif (Supplemental Fig 2). These are VIN hallmarks (Vitale and Chrispeels, 1992; Balk and de Boer, 1999; Sturm, 1999) which are absent from the two CWINs, OsCWI1 and NtCWIN1, included in the comparison. Furthermore, GhVIN1 displayed five distinctive amino acids (indicated by arrows in Supplemental Fig 2) that are highly conserved in VINs but are different from that in CWINs (see Ji et al., 2005).

Consistently, phylogenetic analyses revealed that GhVIN1 was clustered in the plant VIN group, which is evolutionarily close to plant CWINs, but distant from plant CINs, yeast invertase and bacterial invertases. The calculated isoelectric point (pI) of GhVIN1 was 4.7, falling to the range of all VINs; whereas CWINs typically have pI values above 7.0 (Supplemental Fig 3; Sturm, 1999).

To visualize the subcellular location of GhVIN1 in vivo, a 35S:GhVIN1-GFP fusion construct was transformed into Arabidopsis in comparison with that transformed with 35S:GFP control construct. Imaging analyses revealed distinctive patterns of fluorescent signals between root cells from the resultant two classes of transgenic plants. Figure 3A showed circular pattern of GhVIN1-GFP signals in small vacuoles in meristematic cells close to the root tip (arrowheads) and in large vacuoles of cells at the elongation zone (arrows). By contrast, transformation with 35S:GFP construct displayed fluorescent signals throughout the cells including nuclei (filled dots indicated by arrowheads) and cytoplasm adjacent to cell wall that appeared in rectangle shape (arrows in Fig 3B). The multiple small ring-like shapes of GhVIN1-GFP in the meristematic region (arrowheads in Fig 3A) is in agreement with features of dividing cells each of which typically contains small vacuoles. Subsequent cell elongation is characterized by development of a large central vacuole as indicated by the much enlarged circular GhVIN1-GFP signals (arrows in Fig 3A).
To confirm its vacuolar localization, a GhVIN1:RFP fusion construct was co-bombarded with a tonoplast magnesium transporter, ShMTP, fused with GFP (Delhaize et al., 2003) into onion epidermis. Since these cells were heavily vacuolated, the tonoplast would be pushed closely against the plasma membrane and the cell wall. To eliminate the possible ambiguity of signals in intra- and extra-cellular spaces, bombarded samples were plasmolysed before confocal imaging. The plasmolysis allowed the protoplasm to be pulled away from the cell wall that exhibited no GhVIN1:RFP signals (Figs 3D and E). In contrast, the GhVIN1 signals were clearly detected in the large vacuole (Figs 3C to E). Localization of the tonoplast marker, ShMTP:GFP, was shown in Figures 3F to H. Significantly, when GhVIN1:RFP and ShMTP:GFP were co-expressed in one epidermal cell (Figs 3I and J), their colocalization was evident in the fringe of vacuole with GhVIN1:RFP signal dispersed throughout the vacuole while ShMTP:GFP more congregated in the tonoplast as expected (Fig 3K).

Ectopic expression of GhVIN1 in Arabidopsis complements short-root phenotype in a VIN knockout mutant and enhances root cell elongation in wild-type plants

To determine whether GhVIN1 functions as a VIN in planta, complementation experiments were performed on an Arabidopsis knockout (KO) line for a VIN gene. Arabidopsis genome contains two genes of vacuolar invertase, Atβfruct3 (At1g62260, AtVIN1) and Atβfruct4 (At1g12240, AtVIN2). T-DNA insertion in the first exon of Atβfruct4 (Fig 4A) resulted in a short-root phenotype (Sergeeva et al., 2006), hence, representing an ideal system for testing the functionality of GhVIN1. Here, a 35S:GhVIN1 over-expression (OE) construct was transformed into the homozygous KO line (inv). The T-DNA insertion in Atβfruct4 was confirmed by PCR analysis (Fig 4B), as well as by RT-PCR analysis using Atβfruct4 specific primers for the absence of Atβfruct4 mRNA (Fig 4C,
second panel). By contrast, the mRNA of the second *Arabidopsis* VIN gene, *Atβfruct3*, was
detected in both the wild-type and the mutant background (Fig 4C first panel), indicating the
insertion is specific to *Atβfruct4*. Two homozygous complementation lines, C7 3-2 and C14 2-1,
showing strong and weak expression of *GhVIN1* mRNA, respectively (Fig 4C, third panel), rescued
the shoot-root phenotype fully and partially as compared to wild-type when grown on ½ MS (no
sugar) medium; while a null segregant (C5 null) exhibited a similar root length with the *inv* mutant
(*inv*-7) (Fig 4D).

The expression of *GhVIN1* in the mutant background (C7 3-2 and C14 2-1) also recovered root
cortex cell length (Fig 4E). Enzyme assay was conducted to verify if the observed complementation
was due to the increase of VIN activity. Figure 4F shows VIN activity was restored fully to the
wild-type level in the complemented line, C7 3-2 and partially in C14 2-1. By contrast, the CWIN
activity was not affected in the *inv*-7 or in the complemented lines (Fig 4F).

In parallel to the complementation study, the *GhVIN1*-OE construct was also introduced into
wild-type *Arabidopsis* to examine if any positive impact on root growth. *GhVIN1* expression and its
absence were confirmed, respectively, in homozygous over-expression lines (OE 2-10 and OE 5-8)
and in a null segregant, OE null (Supplemental Fig 4A). The over-expression increased root length
by 15-20% and root cortex cell length by 20-30% (Supplemental Figs 4B and C) when grown on
MS medium with 3% sucrose. It also increased root hair length in the stronger expresser of OE 5-8,
where strong VIN activity appeared in the vacuoles (indicated as blue signals in Supplemental Figs
4E and G as compared to the negative control in Fig 4F), and ~ 60% root hairs were longer than 201
µm compared with only 20% and 32% of hairs at this size in the wild-type and null (Supplemental
Fig 4D). The expression of *GhVIN1* resulted in higher activities of VIN but not CWIN
(Supplemental Fig 4H) or CIN (data not shown). Apart from the impact on root elongation no other
visible phenotype was observed in both complementation and over-expression lines. To gain some insights into the GhVIN1-mediated effect on Arabidopsis root elongation, sugar levels were assayed in roots. The analysis revealed lower hexoses and higher sucrose levels in the KO mutant (inv-7) as compared to the wild-type plants. The ectopic expression of GhVIN1 recovered and increased hexose level, thus, the ratio of hexose to sucrose concentration, in the mutant and wild-type background of C14 2-1 and OE 5-8, respectively (Figs 5A and B).

Expression of GhVIN1 correlates with vacuolar invertase activities and cotton fiber elongation

The above analyses (Fig 4 and Supplemental Fig 4) established that GhVIN1 function as a VIN in planta. Further studies were therefore conducted to explore the role of GhVIN1 in cotton fiber elongation. Semi-quantitative RT-PCR analyses showed that the GhVIN1 mRNA was highly expressed in 10-d fibers and seeds, but was barely detectable in roots, stems and cotyledons (Fig 6A). To determine if GhVIN1 is expressed in fiber initials early in elongation, In situ hybridization experiments were conducted on ovule or seed sections. At -2 DAA, prior to fiber initiation (Fig 6B), no GhVIN1 mRNA was detected in ovule epidermis (Fig 6D) in comparison to the sense control (Fig 6C). By contrast, GhVIN1 mRNA signals became evident in the young fiber cells (Fig 6G) as compared to the sense control (Fig 6F) at 0 DAA when fibers are initiating from the seed epidermis as shown by toluidine blue staining (Fig 6E). Importantly, the GhVIN1 mRNA signals were much stronger in the initiating fibers than that in the adjacent non-differentiated epidermal cells or underlying seed coat cells (Fig 6G), which is consistent with that of histochemical staining for invertase activity (see Fig 1B and suppl Fig 1).

Developmentally, GhVIN1 transcript was more abundant in fibers at the fast-elongation phase of 5-10 DAA than that at the slow elongation phase of 15-20 DAA (Fig 6H). Genotypically,
GhVIN1 was expressed more in faster elongating fibers from Gb than that from Gh with slower-elongating fibers (Fig 6I, but also see Fig 2 C). Overall, these expression patterns of GhVIN1 correlates with the changes of fiber VIN activity and elongation rate (see Figs 1 and 2), suggesting GhVIN1 may be a major VIN gene expressed in cotton fiber. Consistent with this assertion, TBLASTN and BLASTN search of cotton EST Contig database (http://www.cottondb.org) using GhVIN1 as a query showed that ~ 90% of the GhVIN1 ESTs were detected in cotton fibers and ovules with the remaining expressed in other tissues.

Silencing and over-expressing GhVIN1 via bombardment reduced and enhanced fiber elongation, respectively.

Finally, to assess the physiological role of GhVIN1 in cotton fiber, we made GhVIN1 RNAi silencing (RNAi) and over-expression (OE) constructs driven by a RDL promoter that is active in cotton fiber and seed early in development (Wang et al., 2004). Both constructs harbored a 35S:GUS reporter gene, thus allowing transformed fiber cells to be visually identified. A RDL:GUS only construct was used as a void-vector control.

Figures 7A and B show evident GUS signals in some fiber cells after 5-d incubation of seed in liquid medium following bombardment at 1 DAA with the RNAi and OE constructs, respectively. This indicates the success of transgene expression in those fibers. The impact of GhVIN1 RNAi silencing and over-expression on fiber VIN activity and elongation was assessed by comparing the transformed fibers identified by GUS blue with adjacent non-transformed fiber cells. The position effect on fiber growth was taken into account by selecting transformed and adjacent untransformed fibers only at the chalazal region of the seed where fibers typically grow well and uniformly as compared to that in the micropyle end (Ruan, 2005). The randomization of the transformed and
untransformed cells in the region further minimized the potential positional effect on the
comparison (see below).

In total, 50 and 36 transformed fibers derived from 25 and 27 cotton seeds, along with equivalent
number of adjacent untransformed cells, were identified and measured for fiber length and enzyme
activity after bombarded with RNAi and OE constructs, respectively (see Materials and methods for
details). Figure 7C shows that, in comparison to those untransformed fibers, transforming with
GhVIN1 RNAi construct reduced VIN activity by 33%, which led to a reduced fiber length (Fig 7D)
On the other hand, fibers expressing GhVIN1 OE construct increased VIN activity by 45% (Fig 7C),
resulting in an increased fiber length (Fig 7D). In contrast, transforming with the void-vector did
not evoke a statistical difference as compared to the non-transformed cells in either VIN activity or
fiber length (Data not shown). We further plotted the fiber VIN activities with fiber length derived
from transformed and untransformed fibers bombarded with the above described constructs. The
analysis revealed a strong correlation (regression coefficient $R^2 = 0.9696$) between changes in VIN
activity and fiber length. By contrast, activities of CIN and CWIN displayed little correlation with
fiber length with $R^2$ value of only 0.0541 and 0.0574, respectively, consistent with previous
observations (Figs 1 and 2). These observations indicate that transformation with *GhVIN1* RNAi or
OE construct affects VIN but not CWIN or CIN activities in cotton fibers.

**Discussion**

*Rapid fiber elongation requires high activity of VIN, probably mediated by *GhVIN1***

We present here several lines of evidence that high activity of VIN contributes to rapid cotton fiber
elongation. Developmentally, VIN activity (Fig 1) and *GhVIN1* transcript level (Fig 6H) was higher
at the rapid expansion phase of 5 and 10 DAA and became evidently lower at the slow elongation
phase of 15-20 DAA. Consistent with the potential role of VIN in fiber elongation, a fiberless
mutant did not exhibit VIN activity in its seed epidermis (Supplementary Fig 1). Genotypically,
higher fiber VIN activity (Fig 2A) and GhVIN1 mRNA level (Fig 6I) correlate with faster fiber
elongation rate (Fig 2C). Importantly, the correlation with fiber elongation is specific to activities of
VIN but not CWIN or CIN (Figs 1 and 2), highlighting the role of VIN in this cell expansion
process. Finally, the causality between VIN and fiber elongation was shown in bombardment
experiments, where transformation with GhVIN1-silencing or over-expression constructs reduced or
enhanced, respectively, VIN activity and fiber elongation (Fig 7). Collectively the data show that
VIN likely plays an important role in cotton fiber elongation.

The notion that GhVIN1 is probably a major vacuolar invertase underpinning the observed VIN
activity in cotton fiber is supported not only by developmental and genotypic correlation between
GhVIN1 transcript level (Fig 6) and VIN activity (Figs 1 and 2) as discussed above but also by the
finding that (i) GhVIN1 was mainly expressed in 10-d fiber and seed with little expression in other
tissues examined (Fig 6A) (ii) GhVIN1 mRNA was abundant in initiating fibers but not in the
adjacent normal epidermal cells nor in ovule epidermis before fiber initiation (Figs 6B-G) and (iii)
~90% GhVIN1 ESTs were detected in cotton fiber and ovule (see Results). Our assertion that
GhVIN1 encodes a functional VIN protein comes from observations that (i) GhVIN1:GFP or :RFP
is localized to the vacuole (Fig 3) and (ii) expression of GhVIN1 in Arabidopsis inv mutant
recovered the VIN activity to wild-type level and consequently complemented the short-root
phenotype (Fig 4).

The above findings are of significance for two reasons. First, VIN has long been considered as a
key player in regulating plant cell expansion (Tang et al., 1999; Andersen et al., 2002; Ruan et al.,
2008). However, experimental evidence directly supporting this hypothesis is lacking due to, in part,
the complexity of multicellular plant tissues (see Ruan et al., 2010). Here we provide an example
that higher activity of VIN activity is likely required for rapid elongation of the single-celled cotton
fibers, taking advantage of its accessibility and extraordinary magnitude of cell expansion (Ruan,
2007). Second, despite the fact that VIN cDNAs have been cloned from a wide range of plant
species, to our knowledge, there have been no reports thus far that any isolated VIN proteins
function in vivo in its native or foreign plants and impact on cell growth. Thus, our demonstration
that GhVIN1 was localized to vacuole (Fig 3) and able to complement the short root phenotype of a
vin knockout line in Arabidopsis (Fig 4) and promoted cotton fiber elongation (Fig 7) represents an
important advance in understanding the functionality of VIN in higher plants.

VIN promotes cotton fiber elongation most likely through osmotic regulation
The fiber cells expand rapidly through the concerted action of turgor pressure and cell wall
relaxation (Ruan et al., 2000), where the vacuole occupies > 90% of the cell volume (Ruan et al.,
2001). The expansion of the vacuoles, hence, fibers, requires efficient influx of water driven by the
accumulation of osmotic solutes. By hydrolyzing sucrose into glucose and fructose, VIN doubles
the osmotic contribution of sucrose, thus has the potential to positively impact on cell turgor.

In this context, glucose and fructose concentrations (Fig 2B) account for about ~50% and 40% of
sap osmolality in10-d fiber from Gb and Gh, respectively, thus representing major osmotic solutes
in fibers from both genotypes. The hexose and sucrose concentrations are about 37 mM and 1.5 mM
higher in the faster elongating 10-fibers from Gb than that from Gh (Figs 2B). This translates into a
difference of ~ -0.1 MPa osmotic potential using the equivalence 2.48 MPa per Osm kg⁻¹
(Schmalstig and Cosgrove, 1988), which would constitutes a 0.1 MPa higher cell turgor in 10-d
fibers from Gb than that of Gh since fiber water potentials are virtually identical between the two
genotypes (Fang and Ruan, unpublished data). This degree of turgor increment could significantly enhance cotton fiber elongation (Ruan et al., 2001) as evidenced by the faster elongation observed in the Gb relative to Gh from 5 DAA onwards (Fig 2C) when VIN activity was significantly higher in the former than the latter (Fig 2A).

It seems paradox that the fiber VIN activity dropped after 5 DAA (Fig 1D), whereas rapidest fiber elongation has been observed to occur from 10 to 15 DAA (Ruan et al., 2001). Here, despite its decrease after 5-d, VIN activity remained ~ two-fold higher in 10-d fiber than that at 15-20 DAA (Fig 1D), hence could promote fiber elongation from 10-15 DAA through osmotic regulation as discussed above. This possibility is indicated by the higher hexose levels in 10-d fiber as compared to that at 15 DAA (Fig 2B). Moreover, other factors may also contribute to cell expansion. In this regard, cell expansion is regulated, in part, by transcellular hydraulic conductance controlled by activity of aquaporins (Maurel et al. 2009). We have recently observed higher expression of two tonoplast intrinsic protein (TIPs) in cotton fiber at 10 DAA as compared to that at 5 and 15 DAA (Jones, Patrick and Ruan unpublished data). The high level expression of these AQPs may facilitate influx of water, hence contribute to the rapid elongation of fibers at 10-15 DAA.

It is of interest to note the higher sucrose level in 10-d fiber from the genotype, Gb, as compared to that of Gh (Fig 2B), a phenomenon appeared to be inconsistent with the higher VIN activity in fibers from Gb. The underlying cause for this disparity is unknown. One possibility is that higher VIN activity in Gb fibers might be coupled with up-regulation of putative tonoplast-bound sucrose transporters (Ruan et al., 2010), which may enhance the influx of sucrose into the vacuole, contributing to the observed higher concentration (Fig 2B).
VIN regulates Arabidopsis root elongation independent of osmotic regulation

Our finding that the cotton GhVIN1 complemented the short-root phenotype of a vin knockout line in Arabidopsis concurred with a previous report by Sergeeva et al., (2006). Notably, once over-expressed in wild-type Arabidopsis, GhVIN1 enhanced root elongation and root hair size (Supplemental Fig 4), indicating normal growth of Arabidopsis root is limited by VIN activity and the utility of GhVIN1 for improving root development by using genetic engineering approach.

Regarding to the mechanism of VIN-regulated Arabidopsis root elongation, it has been proposed that VIN may control the process through osmotic regulation (e.g. Sergeeva et al., 2006). This possibility, however, can now be ruled out based on the following analyses. First, the combined hexose and sucrose concentrations in Arabidopsis roots are extremely low, only at ~2.5, 2.2 and 3.2 mM in the wild-type, vin knockout and GhVIN1-overexpression lines, respectively (Fig 5). This amount of sugars accounts for only 1.6%, 1.0% and 2.1% of the total osmolality measured in the respective lines, rendering their osmotic contribution negligible. In fact, the root sap osmolalities were 159±2.0, 215±1.5 and 149±0.5 Osm kg⁻¹ in the wild-type, vin knockout and GhVIN1-overexpression lines, respectively, which in turn exhibit short, medium and long root length (Fig 4 and Supplemental Fig 4). These findings contradict to the hypothesis of VIN-mediated osmotic regulation of Arabidopsis root elongation.

Cell expansion depends on relaxation of cell wall matrix once cell turgor is above a threshold value. This raises a possibility that VIN may promote Arabidopsis root elongation by impacting on cell wall extensibility. To this end, mutation of a wall-associated receptor-like kinase (WAK) reduces VIN activity and cell elongation in Arabidopsis (Kohorn et al., 2006), suggesting a link between cell wall expansion signaling and VIN activity. It remains to be tested whether this is the case in the vin knockout or GhVIN1 over-expressed line, in particular, whether changes in VIN
activity in the roots of these Arabidopsis lines altered expression/activity of WAK. Alternatively, VIN may regulate Arabidopsis root elongation through glucose-mediated effect on auxin signaling as suggest by a recent report (Mishra et al., 2009).

Finally, the VIN gene Atβfruct4 (AtvacINV2) is also expressed in tissues other than roots (Kohorn et al., 2006), thus mutation of this gene may affect sugar metabolism in those tissues which may indirectly impact on root elongation. The scenario may also apply to the GhVIN1 over-expression lines as the construct was under the control of a constitutive 35S promoter. In this context, hexose and sucrose levels from mature leaves of the mutant plants were about 30% lower and 25% higher, respectively than that the wild-type whereas over-expression of GhVIN1 increased hexose level by ~33% in the mature leaves with no effect on sucrose levels (Supplemental Fig 5). These changes might affect phloem loading and sucrose import to sinks such as roots, thereby affecting their elongation as indicated by mathematic modeling (Naegle et al., 2010).

In conclusion, research presented here provides new insights into the roles of vacuolar invertase (VIN) in plant cell expansion. The data obtained show (i) Rapid fiber elongation requires high activity of VIN, probably mediated by GhVIN1 and (ii) VIN controls cotton fiber and Arabidopsis root elongation through osmotic dependent and independent pathway, respectively. The study provides an example that a given protein (e.g. VIN) could control similar developmental process such as cell expansion through different mechanisms in different cell types.
Materials and Methods

Plant materials

Unless otherwise specified, the cotton genotypes selected for comparative studies were two tetraploid lines, *Gossypium hirsutum* L. var Coker 315 (*Gh*) and *Gossypium barbadense* L. cv. Xinhai 17 (*Gb*), which were from Y-L Ruan’s lab in Newcastle. The Xu142 fuzzless-lintless mutant and its wild-type Xu142 were provided by T-Z Zhang at Nanjing Agricultural University. The plants were grown in a glasshouse at 28 °C with a photoperiod of 14 h light and 10 h dark. Fruit age was determined by tagging the flowering at anthesis. All genotypes were grown in parallel under the same conditions for harvesting samples to compare invertase activity, sugar content, and fiber length and *GhVIN* mRNA levels at specified time points.

*Arabidopsis thaliana* (ecotype Columbia) seeds were surface-sterilized and germinated on either a medium with half MS (Murashige and Skoog, 1962) strength without sugar for complementation study in an *inv* mutant or with MS supplemented with 3% sucrose for over-expression of *GhVIN1* in the wild-type plants (see below). The seedlings were transferred onto a square plate with the same medium at the 5th day after jarovization, and grown vertically for 6 days before harvest for RNA extraction and measurement of root and root cell length and assay of sugar level and enzyme activities. Homozygous lines at T3 generation were used for both complementation and over-expression studies.

Invertase activity assay and localization and sugar measurement

Fresh tissues were immediately frozen and ground to powder in liquid nitrogen, homogenized three times with 1.5ml extraction buffer (see Tomlinson *et al.*, 2004) in total, and centrifuged at 14000 g at 4 °C for 5 min. The supernatant were then used for measurement of CIN and VIN activities and
the pellet was re-suspended by 1.5ml extraction buffer for CWIN activity assay, according to Tomlinson et al., (2004).

For invertase activity assay in transgenic cotton fibers after bombardment, fibers were detached from cotton seeds on ice following GUS staining and photographing (for measuring fiber length, see below). The transformed (indicated by GUS blue) and adjacent non-transformed fibers were collected for assay. About 10-20 fibers for each of the two groups were sampled from 8-10 seeds as one biological sample for VIN activity assay. The results of three biological replicates were used for statistic analysis.

Sugar content was measured enzymatically as described by Stitt et al. (1989).

For invertase activity and sugar content assay, each biological replicate consisted of three technical replicates. These experiments were repeated at least three times and similar results were obtained. One representative set of data was used for analyses described in the paper.

For in situ localization of invertase activity, cotton ovules/seeds at -1, 0 and 1 DAA were collected on ice and fixed in 2% (w/v) paraformaldehyde with 2% (w/v) polyvinylpyrrolidone 40 and 1mM DTT (pH 7.0) at 4°C overnight. The materials were then rinsed by 8 × 15 min to remove soluble sugars from the apoplasm. Invertase activity was stained according to Doehlert and Felker (1987) and Sergeeva and Vreugdenhil (2002) with some modifications: The samples were incubated in 1.0 ml of reaction medium [33mM sodium phosphate buffer at pH6.0, 25 units of glucose oxidase, 0.014% (w/v) phenazine methosulfate, 0.029% (w/v) nitroblue tetrazolium, and 1% sucrose] in at 30°C for 1h. In control reactions, sucrose was omitted. Note, the assay does not differentiate invertase activity from different sub-cellular compartments.
Measurement of cotton fiber length, Arabidopsis root cell and root-hair length and sap osmolality

For fiber length comparison between genotypes Gb and Gh, the fibers were free-hand harvested from seed at specified stages and measured after relaxing and straightening fibers according to Schubert et al. (1973). For measuring fiber length of bombarded cotton seeds, free-hand sections were cut after GUS staining and then photographed under dissecting microscopy (Stemi 2000-C, Zeiss). The length of transformed fibers (blue by GUS staining) was measured using Image J program (http://rsb.info.nih.gov/ij/). For comparison, 2-3 untransformed fibers adjacent to the blue fiber(s) were also measured. To minimize positional effect, fibers for enzyme assay and length measurement were all harvested from the chalazal end of the seeds.

Arabidopsis seedlings were fixed in FAA solution for 16 to 48 hours at 4 ºC, and then transferred into clear solution containing 8 g chloral hydrate and 2ml glycerol and 2 ml distilled water for at least 1 hour. Root samples were microscopically examined (Olympus BX51 wide-field microscope with differential interference contrast) and photographed. The lengths of the cortex cells and root-hairs located in the middle of root-hair zones were measured using Image J program. About 120 cells were measured from six biological replicates (seedlings) for each case (see Fig 4E).

Sap osmolality of cotton fiber and Arabidopsis root was measured using a vapor pressure osmometer (Wescor 5520). For each biological replicate, about 50 mg of fresh sample were snap-frozen in liquid nitrogen and then thawed at 4 ºC. Thereafter, the sample was spun at 5000 g for 1 min to collect the sap. Ten micro-liter of the resultant supernatant was collected for measurement of sap osmolality. Three biological replicates, each with three technical replicates, were used to compute the osmolality for each line or tissue.
Cloning and sequence analyses of GhVIN1

A Lambda Zap Expression cDNA Library was prepared from mRNA isolated from developing cotton fiber and seeds at -3 to 25 DAA. Random sequencing identified one putative vacuolar invertase clone, designated GhVIN1. Full length sequence of this cotton invertase was generated from fiber cDNA by using PCR with gene-specific primers of GhVIN1-FL-XF and GhVIN1-FL-XR.

Computational analyses were performed using the GCG SeqWeb program (version 2.0.2; Accerlyx). For phylogenetic analysis, the deduced amino acid sequences were sent to CLUSTALW (http://clustalw.genome.jp), from which results were exported to the TreeView program (Page, 1996) for construction.

Subcellular localization of GhVIN1-GFP or -RFP

The pGhVIN1-GFP construct was made as follows: the full length GhVIN1 cDNA without stop codon was amplified using primers of GhVIN1-FL-PXF and GhVIN1-FL-BR. The GhVIN1 cDNA was ligated into pEGFP vector upstream of EGFP. Digestion of the vector with XbaI yielded GhVIN1-GFP fusion fragment, which was then cloned into pCAMBIA1300 vector downstream of 35S promoter.

For constructing pGhVIN1-RFP vector, the full length fragment of GhVIN1 was released by digestion with PstI and BamHI and cloned into pGDR (Goodin et al., 2002) downstream of DsRed2 (RFP) by double digestion with Sal I and BamH I. The construct was under the control of 35S promoter.

The GhVIN1-GFP and -GFP control constructs were transformed into Agrobacterium
*tumefaciens* strain GV3101 for stable transformation into *Arabidopsis* through floral dip method.

Transient expression of GhVIN1-GFP or -RFP fusion proteins in onion (*Allium cepa*) epidermal

cells was performed with a PDS-1000/He Biolistic particle delivery system (Bio-Rad) as described

by Kikkert (1993). GFP and RFP-expressing cells were detected with a confocal laser scanning

microscope (Zeiss LSM 510 META) with argon laser excitation wavelength of 488 nm (GFP) or

543 nm (RFP).



**GhVIN1 over-expression construct for transformation of Arabidopsis**

Full length *GhVIN1* cDNA was amplified for sub-cloning into pCAMBIA 1300 vector downstream

of a 35S promoter. The *GhVIN1*-OE vector was introduced into *Arabidopsis* via

*Aegrobacterium*-mediated transformation using the floral dip method.

Primers used for making the above constructs were listed in Supplemental Table 2.



**In situ hybridization**

In situ hybridization analyses of 0 and 1 DAA cotton seeds were performed according to Xu *et al.*

(2008). A 452bp *GhVIN1* fragment, ranging from 1484 bp to 1935 bp downstream of start codon,

was amplified with GhVIN1-1521F and GhVIN1-FL-XR primers (Supplemental table 2), cloned

into pBluescript II SK and linearized with BamHI and XbaI for making sense and antisense probes,

respectively.



**Semi-quantitative reverse transcription (RT)-PCR analyses**

Total RNA was isolated from cotton fiber and other tissues according to Ruan *et al.*, (1997). For

each reaction, 1μg RNA was reverse-transcribed to cDNA with an oligo (dT) primer. Gene specific
primers (GhVIN1-RT) were designed to amplify a 684 bp fragment of GhVIN1. The cotton 18srRNA gene was used as an internal control, amplified with Gh18srRNA gene RT primers.

For RT-PCR analyses in Arabidopsis, total RNA was isolated using Trizol from Invitrogen and reverse-transcribed as previously described. Atβfruct3 and Atβfruct4 transcript was determined using Atβfruct3-RT and Atβfruct4-RT primers, with Arabidopsis tubulin1 (AtTUB) as an internal control, amplified with AtTUB-RT primers.

All PCR conditions were optimized, and mentioned in Supplemental Table 2.

GhVIN1 RNAi and over-expression constructs for bombardment of cultured cotton seed

Void-vector for particle bombardment, pRN-1301, was constructed by transferring a 302bp fiber-specific RDL promoter (Wang et al., 2004) (HindIII and PstI) and a 253bp NOS terminator (EcoRI and XhoI) into the polylinker site of pCAMBIA1301 vector (CAMBIA, Canberra, Australia), which carries a CaMV 35S: GUS: NOS terminator cassette in opposite direction but with hygromycin resistance gene removed.

For constructing the RNAi vector (pRN-RNAi-1301), a 185 bp GhVIN1 cDNA fragment was amplified with GhVIN1-S and GhVIN1-AS primers and ligated into pHANNIBAL (NCBI accession number: AJ311872) in an invert-repeat orientation, franked by a 740bp intron, downstream of a 35S promoter. The promoter was then substituted by RDL promoter in the SacI-XhoI sites. The RDL-RNAi gene cassette was then ligated into pHB by digestion with SacI and XbaI. pHB is an intermediate vector modified from pRN-1301 (originated from X-Y Chen at Shanghai Institute of Biological Sciences). The RDL-RNAi gene cassette was released by digestion with Hind III and Xba I, and ligated into pRN-1301.

The GhVIN1 over-expressing vector (pRN-OE-1301) was constructed by ligating full length
GhVIN1 cDNA into pRN-1301 in XbaI site, downstream of RDL promoter.

Cotton ovule culture and particle bombardment was conducted using the method described by Kim et al., (2002) with the following modifications: cotton seeds at 1 DAA were bombarded under 1100 psi Helium pressure and incubated in solid BT medium for 2 d after bombardment. Thereafter, the cotton seeds were transferred onto liquid BT medium supplemented with 10 µM IAA and 0.05 µM GA3 for 5 d before staining for GUS activity, measurement of enzyme activity, and fiber length. In each case, transformed fibers were identified by GUS staining (see Figs 7 A to C). Transformed and adjacent untransformed fibers were dissected separately from the seed for measurements.

Statistic analysis

Data were analyzed by randomization one-way analysis of variance test (ANOVA) for analyses of invertase activity across multiple time points during fiber elongation in Gh and for comparison among Arabidopsis wild-type, inv mutant, GhVIN1 complemented lines and over-expressed lines for root length, root cell length, invertase activity and sugar content, Student-t test was performed for (i) cotton genotypic comparison between Gb and Gh for invertase activity, sugar content and fiber length, and (ii) comparison between transformed and adjacent untransformed fibers after bombardment for fiber length and invertase activity. Means were compared using LSD tests in all analysis mentioned above. All statistical calculations were performed using DPS© package (version 8.01; Tang and Feng, 2007).

Accession Number

The Genbank accession number of GhVIN1 is FJ915120.
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Support information - short legends:

Supplemental Figure 1. In situ localization and assay of invertase activity in a fiberless cotton seed mutant and its wild-type.

Supplemental Figure 2. Alignment of GhVIN1 sequence with three plant VINs (LeVIN1, OsINV3 and ZmIvr1) and two CWINs (OsCWI1 and NtCWIN1).

Supplemental Figure 3. Phylogenetic analysis of GhVIN1 with invertases from other species.

Supplemental Figure 4. Impact of over-expression of GhVIN1 on Arabidopsis root elongation.

Supplemental Figure 5. Sugar levels in mature leaves of vin-knockout, GhVIN1-overexpressed and wild-type Arabidopsis lines.

Supplemental Table 1. Genbank accession numbers of invertase genes used in Supplemental Figures 3.

Supplemental Table 2. Primers and PCR conditions used in this study.
FIGURE LEGENDS

Figure 1. Invertase activity assay in cotton fiber and other tissues.

(A) Invertase activity in different cotton tissues. R, root; S, stem; C, cotyledon; L, source leaf. F, Sc and Ft represent 10-d fiber, seed coat and filial tissue, respectively; CIN, cytoplasm invertase; VIN, vacuolar invertase; CWIN, cell wall invertase.

(B) Localization of invertase activity in whole cotton ovule and seed showing dark blue signals of invertase activity present in fiber-enriched epidermis at 0 and 1 DAA seeds but not in epidermis of -1 DAA ovule prior to fiber initiation (see Results for more detail). Bar= 1 mm

(C) Invertase activity assay in ovule/seed extracts from -1 to +1 DAA.

(D) Invertase activity in fibers during elongation.

Each value in (A), (C) and (D) is the mean ± SE of at least four biological replicates. Different letters in (C) indicate significantly different at $P \leq 0.05$ according to randomization one-way ANOVA test.

Figure 2. Genotypic comparison of invertase activity, sugar content and fiber length.

(A) Invertase activity during fiber elongation.

(B) Sugar content in elongating fibers at 10 and 15 DAA in Gb and Gh.

(C) Kinetics of fiber elongation. Fibers from genotype Gb are significantly longer than those from Gh at 10, 15 and 20 DAA. The data were reproduced from Li et al 2010 and were collected from the same batch of plants used for the current study.

Each value in (A) and (B) is the mean ± SE of at least three biological replicates. Each value in (C) is the mean ± SE of twelve biological replicates. Asterisks indicate significant difference (student-t
test, * \( P \leq 0.05; ** P \leq 0.01 \) between fibers from genotype \( Gb \) and \( Gh \) at each tested time-point.

**Figure 3.** Subcellular localization of GhVIN1-GFP and -RFP fusion proteins.

(A) and (B) Stable expression of GhVIN1-GFP and -GFP, respectively, in *Arabidopsis* root. Note in (A) circular pattern of GhVIN1-GFP signals in small vacuoles in meristematic cells close to the root tip (arrowheads) and in large vacuoles in cells at the elongation zone (arrows). By contrast, transformation with 35S:GFP construct in (B) showed signals throughout the cells including nuclei (arrowheads) and cytoplasm adjacent to cell wall that appeared in rectangle shape (arrows).

(C), (D) and (E) GhVIN1-RFP expression in plasmolysed onion epidermal cells after bombardment, showing signals of GhVIN1 in shrunken vacuole (C), the same image in bright field (D) and their merged image (E). Note, the plasma membranes (PM) were pulled away from the cell wall (CW) and the GhVIN1 signals were clearly confined within the vacuole (Vac, D and E).

(F), (G) and (H) Transient expression of ShMTP-GFP, a tonoplast marker (*Delhaize et al.*, 2003) in plasmolysed onion epidermal cells.

(I), (J) and (K) Colocalization of GhVIN1-RFP and ShMTP-GFP in an onion epidermal cell after co-bombardment. Note, the GhVIN1-RFP signals were distributed throughout the vacuole (I), whereas ShMTP-GFP signals were concentrated in the tonoplast and regions nearby (J). After overlaying the two images (K), colocalization of GhVIN1-RFP and ShMTP-GFP was evident with the former appeared also inside the vacuole where ShMTP-GFP signals were absent (arrows) as expected.

Bars in (A) and (B) = 20 \( \mu m \); Bars in (C) to (K) = 50 \( \mu m \).
Figure 4. Ectopic expression of GhVIN1 complements short root phenotype in Arabidopsis vacuolar invertase, Atβfruct4, T-DNA insertion mutant.

(A) A schematic presentation of the Atβfruct4 gene with 7 exons (boxed) and 6 introns (lines). Positions of primers used for identification of the T-DNA insertion (BP and RP) or confirmation of the T-DNA absence (LP and RP) were indicated. UTR, un-translated region.

(B) The T-DNA insertion in Atβfruct4 was confirmed by PCR. The inv mutant (inv-7), and two complemented lines of C7 3-2 and C14 2-1 as well as their null segregant (C5 null) contained the T-DNA, which was absent in the wild-type plants.

(C) RT-PCR analysis showed the absence of Atβfruct4 mRNA in inv-7, C5 null, C7 3-2 and C14 2-1, and the presence of GhVIN1 mRNA in lines C7 3-2 and C14 2-1. The mRNA of the other native VIN, Atβfruct3, was present in all the tested lines. AtTUB was used as an internal control.

(D) The short-root phenotype in inv-7 and C5 null was complemented in GhVIN1-expressed line C7 3-2 and C14 2-1. The left and right panels represent root phenotype (bar = 10 mm) and calculated root length (each value is the mean ± SE of eighteen seedlings), respectively.

(E) The short root cell phenotype of inv-7 and C5 null was fully and partially rescued in GhVIN1-complemented lines C7 3-2 and C14 2-1 respectively, compared to the wild-type level. Each value is the mean ± SE of six seedlings. Values in parentheses indicate number of cells calculated for each line.

(F) Assay of invertase activities reveals increased levels of VIN in complemented lines (C7 3-2 and C14 2-1) in comparison with that of the mutant (inv-7) and C5 null. By contrast, the CWIN activities remain almost unaltered among these lines. Each value is the mean ± SE of six biological replicates.
Different letters in (D), (E) and (F) indicate significant differences at $P \leq 0.05$ according to randomization one-way ANOVA test.

**Figure 5.** Impact of ectopic expression of GhVIN1 on *Arabidopsis* sugar levels.

(A) *vin* mutant (*inv*-7) exhibited reduced glucose and fructose level and increased sucrose concentration. Expression of GhVIN1 complemented and increased hexose levels in the *VIN* mutant (C14 2-1) and wild-type (OE 5-8) background, respectively.

(B) Expression of GhVIN1 complemented and increased hexose to sucrose ratio in the *VIN* mutant (C14 2-1) and wild-type (OE 5-8) background, respectively.

Each value is the mean ± SE of at least four biological replicates. Different letters indicate significant differences at $P \leq 0.05$ according to randomization one-way ANOVA test.

**Figure 6.** Transcript level of *GhVIN1* in cotton fiber and other tissues.

(A) Transcript level of *GhVIN1* in root (R), stem (S), cotyledon (C) source leaf (L). F, Sc and Ft represent 10-d fiber, seed coat and filial tissue, respectively.

(B-D) A longitude-section of -2 d cotton ovule stained with toluidine blue (B), hybridized with a sense (C) and an antisense (D) RNA probe for *GhVIN1*. epi, epidermal cells, indicated by arrows; isc, inner seed coat; osc, outer seed coat.

(E-G) A longitude-section of 0 d cotton seed stained with toluidine blue (E), hybridized with a sense (F) and an antisense (G) RNA probe for *GhVIN1*, respectively. Note strong signals of *GhVIN1* mRNA in fibers (f, indicated by arrowheads) as compared to the adjacent epidermal cells (epi, arrow) in (G).

(H) Transcript level of *GhVIN1* in cotton fiber during elongation.
(I) Transcript level of \textit{GhVIN1} was higher in the long fiber genotype \textit{Gb} than that in \textit{Gh}.$^{18}$

18\textit{srRNA} was used as an internal control. Bar in = 20 µm in (B) to (G).

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Figure 7. Impact on cotton fiber elongation by bombarding cultured seeds with RDL:GhVIN1 RNAi or over-expression (OE) constructs linked with GUS reporter gene.

(A) and (B) Representative images of cotton fiber bombarded with \textit{RDL:GhVIN1}-RNAi:GUS (RNAi) and \textit{RDL:GhVIN1}-OE:GUS (OE) constructs, respectively.

Fibers showing transgene expression (indicated by GUS blue) are used for analyses in comparison with adjacent non-transformed fibers.

(C) VIN activities of transformed fibers and their adjacent non-transformed fibers.

(D) Comparison of fiber length between transformed and their adjacent non-transformed fibers.

Bar = 200µm in (A) and (B).

In (C) and (D), each value is the mean ± SE three biological replicates. Asterisks indicate significant differences (student-t test, * \( P \leq 0.05 \); ** \( P \leq 0.01 \)) between transformed and non-transformed fibers.
**Fig 1** Wang et al 2010
**Figure 1.** Invertase activity assay in cotton fiber and other tissues.

(A) Invertase activity in different cotton tissues. R, root; S, stem; C, cotyledon; L, source leaf. F, Sc and Ft represent 10-d fiber, seed coat and filial tissue, respectively; CIN, cytoplasm invertase; VIN, vacuolar invertase; CWIN, cell wall invertase.

(B) Localization of invertase activity in whole cotton ovule and seed showing dark blue signals of invertase activity present in fiber-enriched epidermis at 0 and 1 DAA seeds but not in epidermis of -1 DAA ovule prior to fiber initiation (see Results for more detail). Bar= 1 mm

(C) Invertase activity assay in ovule/seed extracts from -1 to +1 DAA.

(D) Invertase activity in fibers during elongation.

Each value in (A), (C) and (D) is the mean ± SE of at least four biological replicates. Different letters in (C) indicate significantly different at $P \leq 0.05$ according to randomization one-way ANOVA test.
Figure 2. Genotypic comparison of invertase activity, sugar content and fiber length.

(A) Invertase activity during fiber elongation.

(B) Sugar content in elongating fibers at 10 and 15 DAA in Gb and Gh.

(C) Kinetics of fiber elongation. Fibers from genotype Gb are significantly longer than those from Gh at 10, 15 and 20 DAA. The data were reproduced from Li et al (2010) and were collected from the same batch of plants used for the current study.

Each value in (A) and (B) is the mean ± SE of at least three biological replicates. Each value in (C) is the mean ± SE of twelve biological replicates. Asterisks indicate significant difference (student-t test, * P ≤ 0.05; ** P ≤ 0.01) between fibers from genotype Gb and Gh at each tested time-point.
Figure 3. Subcellular localization of GhVIN1-GFP and -RFP fusion proteins.

(A) and (B) Stable expression of GhVIN1-GFP and GFP only, respectively, in *Arabidopsis* root. Note in (A) circular pattern of GhVIN1-GFP signals in small vacuoles in meristematic cells close to the root tip (arrowheads) and in large vacuoles in cells at the elongation zone (arrows). By contrast, transformation with 35S:GFP construct in (B) showed signals throughout the cells including nuclei (arrowheads) and cytoplasm adjacent to cell wall that appeared in rectangle shape (arrows).

(C), (D) and (E) GhVIN1-RFP expression in plasmolysed onion epidermal cells after bombardment, showing signals of GhVIN1 in shrunken vacuole (C), the same image in bright field (D) and their merged image (E). Note, the plasma membranes (PM) were pulled away from the cell wall (CW) and the GhVIN1 signals were clearly confined within the vacuole (Vac, D and E).

(F), (G) and (H) Transient expression of ShMTP-GFP, a tonoplast marker (Delhaize *et al.*, 2003) in plasmolysed onion epidermal cells.

(I), (J) and (K) Colocalization of GhVIN1-RFP and ShMTP-GFP in an onion epidermal cell after co-bombardment. Note, the GhVIN1-RFP signals were distributed throughout the vacuole (I), whereas ShMTP-GFP signals were concentrated in the tonoplast and regions nearby (J). After overlaying the two images (K), colocalization of GhVIN1-RFP and ShMTP-GFP was evident with the former appeared also inside the vacuole where ShMTP-GFP signals were absent (arrows) as expected.

Bars in (A) and (B) = 20 µm; Bars in (C) to (K) = 50 µm.
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**A**

![Diagram of gene structure](image)

**B**

![Southern blot](image)

**D**

![Image of root length](image)

**E**

![Bar graph of root cell length](image)

**F**

![Bar graph of enzyme activity](image)

- **At1g12240** (Atβfruct4)
- **5'UTR**
- **3'UTR**
- **BP**
- **LP**
- **T-DNA**
- **LP/RP (1093bp)**
- **BP/RP (766bp)**
- **inv**
- **inv-7**: C5 null: 63.96 ± 1.22 μm (117)
  - C7 3-2: 75.42 ± 1.64 μm (120)
  - C14 2-1: 70.81 ± 1.29 μm (119)
  - WT: 78.84 ± 1.25 μm (116)
- **WT**
- **GhVIN1**
- **AtTUB**
- **VIN**
- **CWIN**

**Enzyme activity**

- **VIN**: 0.0 a b c d e
- **CWIN**: 0.0 a b c d e

*Figure 4* 

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Figure 4. Ectopic expression of GhVIN1 complements short root phenotype in Arabidopsis vacuolar invertase, Atβfruct4, T-DNA insertion mutant.

(A) A schematic presentation of the Atβfruct4 gene with 7 exons (boxed) and 6 introns (lines). Positions of primers used for identification of the T-DNA insertion (BP and RP) or confirmation of the T-DNA absence (LP and RP) were indicated. UTR, un-translated region.

(B) The T-DNA insertion in Atβfruct4 was confirmed by PCR. The inv mutant (inv-7), and two complemented lines of C7 3-2 and C14 2-1 as well as their null segregant (C5 null) contained the T-DNA, which was absent in the wild-type plants.

(C) RT-PCR analysis showed the absence of Atβfruct4 mRNA in inv-7, C5 null, C7 3-2 and C14 2-1, and the presence of GhVIN1 mRNA in lines C7 3-2 and C14 2-1. The mRNA of other native VIN, Atβfruct3, was expressed in all the tested lines. AtTUB was used as an internal control.

(D) The short-root phenotype in inv-7 and C5 null was complemented in GhVIN1- expressed line C7 3-2 and C14 2-1. The left and right panels represent root phenotype (bar = 10 mm) and calculated root length (each value is the mean ± SE of eighteen seedlings), respectively.

(E) The short root cell phenotype of inv-7 and C5 null was fully and partially rescued in GhVIN1-complemented lines C7 3-2 and C14 2-1 respectively, compared to the wild-type level. Each value is the mean ± SE of six seedlings. Values in parentheses indicate number of cells calculated for each line.

(F) Assay of invertase activities reveals increased levels of VIN in complemented lines (C7 3-2 and C14 2-1) in comparison with that of the mutant (inv-7) and C5 null. By contrast, the CWIN activities remain almost unaltered among these lines. Each value is the mean ± SE of six biological replicates. Different letters in (D), (E) and (F) indicate significant differences at $P \leq 0.05$ according to randomization one-way ANOVA test.
**Figure 5.** Impact of ectopic expression of GhVIN1 on *Arabidopsis* sugar levels.

(A) *vin* mutant (*inv*-7) exhibited reduced glucose and fructose level and increased sucrose concentration. Expression of GhVIN1 complemented and increased hexose levels in the *VIN* mutant (C14 2-1) and wild-type (OE 5-8) background, respectively.

(B) Expression of GhVIN1 complemented and increased hexose to sucrose ratio in the *VIN* mutant (C14 2-1) and wild-type (OE 5-8) background, respectively.

Each value is the mean ± SE of at least four biological replicates. Different letters indicate significant differences at $P \leq 0.05$ according to randomization one-way ANOVA test.
Figure 6. Transcript level of *GhVIN1* in cotton fiber and other tissues.

(A) Transcript level of *GhVIN1* in root (R), stem (S), cotyledon (C) source leaf (L). F, Sc and Ft represent 10-d fiber, seed coat and filial tissue, respectively.

(B-D) A longitude-section of -2 d cotton ovule stained with toluidine blue (B), hybridized with a sense (C) and an antisense (D) RNA probe for *GhVIN1*. epi, epidermal cells, indicated by arrows; isc, inner seed coat; osc, outer seed coat.

(E-G) A longitude-section of 0 d cotton seed stained with toluidine blue (E), hybridized with a sense (F) and an antisense (G) RNA probe for *GhVIN1*, respectively. Note strong signals of *GhVIN1* mRNA in fibers (f, indicated by arrowheads) as compared to the adjacent epidermal cells (epi, arrow) in (G).

(H) Transcript level of *GhVIN1* in cotton fiber during elongation.

(I) Transcript level of *GhVIN1* was higher in the long fiber genotype *Gb* than that in *Gh*. *18srRNA* was used as an internal control. Bar in = 20 µm in (B) to (G).
**Figure 7.** Impact on cotton fiber elongation by bombarding cultured seeds with RDL:GhVIN1 RNAi or over-expression (OE) constructs linked with GUS reporter gene.

(A) and (B) Representative images of cotton fiber bombarded with *RDL:GhVIN1*-RNAi:GUS (RNAi) and *RDL:GhVIN1*-OE:GUS (OE) constructs, respectively. Fibers showing transgene expression (indicated by GUS blue) are used for analyses in comparison with adjacent non-transformed fibers.

(C) VIN activities of transformed fibers and their adjacent non-transformed fibers.

(D) Comparison of fiber length between transformed and their adjacent non-transformed fibers. Bar = 200 µm in (A) and (B). In (C) and (D), each value is the mean ± SE three biological replicates. Asterisks indicate significant differences (student-t test, * P ≤ 0.05; ** P ≤ 0.01) between transformed and non-transformed fibers.