ISSN 1021-4437, Russian Journal of Plant Physiology, 2019, Vol. 66, No. 1, pp. 41–49. © Pleiades Publishing, Ltd., 2019.

RESEARCH PAPERS

Sucrose Synthase (SuSy) Gene Expression: An Indicator for Cotton Fiber Initiation and Early Development[1]

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Received October 6, 2017; revised April 17, 2018; accepted May 7, 2018

Abstract—Cotton (*Gossypium hirsutum* L.) fiber initiation from ovule epidermal cells happen from 0 to 5 DPA, invertase (INV) and sucrose synthase (SuSy) are crucial for fiber initiation, cell expansion, and elongation. We used two commercial cotton varieties, Xuzhou 142-normal wild-type (WT) and Sea Island PimaS-4 (PimaS-4) as controls and compared with three fiber mutants, Xuzhou 142-fl (fl), Xuzhou 142-N (N) and Ligon lintless (Li). SuSy, INV activity, sugars and malate content were measured at fiber cell initiation and early development stage. Increased Susy activity was detected in WT, PimaS-4 and Li ovules at 0 DPA than the fiber mutant lines fl and N. On the other hand, fl mutant showed high sucrose contents than N and Li during 0 to 1 DPA. No significant changes happen in studied cotton lines with respect to INV from 1 to 5 DPA altogether. There was a significant difference in total soluble sugars and malate contents between WT and fl cotton ovules at early elongation stage (5 DPA). The results revealed that SuSy activity at anthesis day indicates the fate of ovule epidermal cells to bulge out and form fiber initials. The reduced SuSy activity in fl ovules at 0 DPA results in a lack of fiber cell initiation and lead to fiberless seed phenotype. The study will pave the way towards unraveling the mechanism of fiber initiation and development by exploring the role of different fiber-related genes.

Keywords: Gossypium hirsutum, sucrose synthase, invertase, cotton fiber development, sugar metabolism, day post anthesis

DOI: 10.1134/S1021443719010023

INTRODUCTION

Cotton fiber is a typical example of a plant trichome that originates from an ovule epidermal cell and undergoes a process of differentiation. The molecular and metabolic mechanisms that govern fiber cell initiation from the ovule epidermis are still unclear. Although research shows that cotton ovule epidermal cells have the potential to develop into fiber cells, in fact, only 25–30% of ovule epidermal cells develop into fibers [1, 2]. Thus, an understanding of the mechanism that discloses why some, but not all, epidermal cells develop into fibers will deepen our knowledge for increasing fiber production using advanced molecular approaches. Anatomically, fiber cell initiation is associated with the spherical protuberance of a cotton ovule epidermal cell on the anthesis day (0 DPA). The mature fiber cell is the result of primary wall extension and secondary wall thickening without cell division. Cotton fibers, being unique cells, show a remarkable capacity for cell wall biosynthesis and enable the study of associated processes. During the process of growth and development, cotton fiber passes through distinct but overlapping stages i.e. initiation, elongation with primary cell wall synthesis, secondary cell wall synthesis, and maturation. The mature fiber cells can reach up to 2.5–3 cm, about 2000–3000 times their diameter [3]. The developing cotton fiber is a highly active sink cell that uses sucrose for its rapid expansion and cellulose synthesis. In fiber cells, sucrose synthase (SuSy) is a major sucrolytic enzyme [4, 5]. SuSy expression plays a different role in fibers, endosperm, embryos and maternal tissues during the development of cottonseed [1, 6].

The appearance of SuSy activity is considered to be the first important signal for the commencement of

[1] The article is published in the original.

Abbreviations: DNS—dinitrosalicylic acid; DPA—days post anthesis; fl—fuzzless-lintless; GhVIN1—*Gossypium hirsutum* vacuolar invertase; INV—invertase; Li—ligon cotton; N—Xuzhou 142-N naked seed; OD—optical density; RNAi—RNA interference; SuSy—sucrose synthase; UDPG—uridine diphosphate glucose; WT—wild type.

fiber initiation [4, 5, 7]. SuSy is often seen in higher plant cells that play a role in the breakdown of sucrose; the resulting products are often involved in respiration and the synthesis of cell wall polysaccharides such as cellulose and starch [8, 9]. SuSy has been found to be involved in different cotton fiber cell initiation process and is an up-regulated signal factor in cellulose synthesis stages [10–12]. It catalyzes the reversible cleavage of sucrose into fructose and UDP-glucose [13–15].

$$\text{Sucrose} + \text{UDP} \xleftrightarrow{\text{SuSy}} \text{UDP-glucose} + \text{Fructose}.$$

UDPG (uridine diphosphate glucose) has been proven to be a direct precursor for the synthesis of cellulose [14, 16, 17]. In the cell membrane of sink cells, UDPG releases glucose and glucose subunits are polymerized into long-chain cellulose in the presence of cellulose synthase.

Invertase (INV), also known as sucrase, hydrolyzes sucrose (major mobile sugar) into glucose and fructose, which play a decisive role in plant metabolism as well as early seed development [18]. The prime mechanism behind this regulation relies on the ability of INV to modify sugar signals by producing glucose instead of UDP-glucose:

$$\text{Sucrose} + \text{H}_2\text{O} \xrightarrow{\text{INV}} \text{Glucose} + \text{Fructose}.$$

Sucrose breakdown can occur in the cell wall (CWINV), in the cytoplasm (CINV), or in the vacuole (VINV). Moreover, SuSy is also localized in the plasma membrane [10]. When sucrose breakdown takes place in the cytoplasm, it results in limited hexose or hexose-based signaling compared to sucrose, which crosses the cell wall and is cleaved by CWINV [19]. Previous studies found that hexoses support cell division and expansion, while sucrose favors differentiation and maturation [17, 20]. Most of the research exploring the mechanism of cotton fiber elongation coincides with the "theory of osmotic regulation" [21, 22]. The accumulation of osmotic active substances produces turgor pressure, which promotes cell elongation. The osmotic pressure of these active substances may be due to the presence of sugars or the active transport of K^+ and malate into the cell's vacuole. The plant cell elongation is the result of a combination of many factors, including cell wall structure, turgor pressure, phospholipid membrane synthesis, cell wall proteins and cellular enzymes.

The main aim of the study was to explore the role of SuSy activity in fiber initiation and early development by characterizing the five different types of cotton materials: Xuzhou 142 (upland cotton) with normal fibers as control, Xuzhou 142-N without fuzz (fuzzless), Xuzhou 142-fl (fuzzless-lintless), Ligon lintless (Li) with very short fibers about 6–8 mm length and Sea Island PimaS-4 cotton. The investigation of enzymatic changes, sucrose, total soluble sugars, and malic acid contents during fiber cell initiation in ovule epidermal cells was carried out using these materials. The study provides strong evidence that SuSy plays a crucial role in fiber cell initiation on the flowering day. It can be inferred from the results of the current study that fiber cell initiation and SuSy activity are coupled to each other. Changes in metabolite levels and the enzymatic activity of SuSy in early development (up to 5 DPA) are more important than INV, while INV is strongly correlated with structural alterations in seeds and seed phenotype. The detailed study of an fl mutant which is fuzzless and lintless due to a mutation of fiber-related genes, including the SuSy gene, provided a clear picture to explore the mechanism of fiber initiation as "an important economic trait" and help genetic breeders to improve fiber productivity to fulfill the requirements of the modern textile industry.

MATERIALS AND METHODS

Plant material. Three cotton (*Gossypium hirsutum* L.) isogenic lines: Xuzhou 142 normal upland cotton (WT) having normal fibers was used as a control, Xuzhou 142-N lacking short fibers (fuzzless) and Xuzhou 142-fl fuzzless-lintless (fl) were used for studying the process of fiber initiation. Ligon lintless (Li) cotton that is defective in fiber cell elongation and PimaS-4 cotton were also taken. Morphological and phenotypic characteristics of cotton lines are shown in Table 1 and Fig. 1. Ovules of all lines before and after flowering (–1, 0, 2 and 5 DPA) were selected and used for determination of sucrose synthase (SuSy) and invertase (INV) activity along with total soluble sugars and malic acid contents. INV activity was also determined from separated fibers of 10 DPA ovules. For determination of sucrose and total soluble sugar content, samples were dried at 60°C.

Determination of SuSy activity. Preparation of crude enzyme extract: fresh cotton ovules (0.5 g) were taken, 2 mL of 50 mM phosphate buffer (2.56 mL 0.6 M Na_2HPO_4, 1.64 mL 0.2 M EDTA-Na_2, 0.75 mL 0.6 M NaH_2PO_4, volume raised up to 100 mL with water) was added followed by crushing for homogenization with chilled pestle and mortar. The slurry was centrifuged at 5°C, 4500 rpm for 20 min and the supernatant was taken into 1 mL of ammonium sulfate precipitation. 50% of saturated sediment was suspended in 0.2 mL of 50 mM phosphate buffer then poured into dialysis bag, placed in 1000 mL of 10 mM phosphate buffer (10.2 mL of 0.6 M Na_2HPO_4, add 6.6 mL of 0.6 M NaH_2PO_4 and 10 mL of 0.2 M EDTA-Na_2 volume raised up to 2000 mL with water), and kept at 0 ~ 4°C

for overnight dialysis with constant volume to 0.5 mL (crude enzyme extract).

Enzyme activity: SuSy activity was measured by taking 0.1 mL of 0.05 M fructose, 0.1 mL UDPG (8.2 mg UDPG, volume raised up to 1 mL with water) 0.1 mL of 0.1 M Tris, 0.05 mL of 10 mM $MgCl_2$ and 0.2 mL crude enzyme extract. The above reaction mixture was placed at 37°C for 30 min and then transferred in a water bath at 100°C for 1 min, centrifuged and the 1 mL supernatant was taken from it. After this, 0.1 mL of 2 N NaOH was added to it and kept in boiling water for 10 min. It was then allowed to cool in the water, and then 3.5 mL 30% HCl was added along with mixing of 1 mL 0.1% resorcinol. The mixture was kept in a water bath for 10 min at 80°C. The reaction was cooled in water and OD was taken at 480 nm with the help of spectrophotometer. In another reaction mixture, about 20 to 200 μg sugar was added and final volume was adjusted to 1 mL with water and then 0.11 mL 2 N NaOH was added. Subsequent steps were taken as mentioned afore, results were measured with different concentrations of sucrose and a standard curve was drawn to calculate the reaction of sucrose sugar. SuSy activity was measured as mg sucrose = mg/(g h).

Determination of sucrose and total soluble sugars. Sucrose content was determined by weighing 50 mg dry cotton ovules in which 3 mL of 80% ethanol was added. The reaction mixture was boiled at 80°C in water bath for 30 min. After heating, the samples were repeatedly centrifuged and the supernatant was taken three times. The final volume was raised up to 10 mL and 0.3 mL volume was drawn into a new tube. Tubes were placed in boiling water bath until the sample became concentrated and reduced to 0.05 to 0.1 mL. After boiling, 0.1 mL 30% KOH was added to the samples and they were then kept in boiling water bath for 10 min after which they were cooled down at room temperature. Anthrone reagent (0.3 mL prepared by adding 150 mg anthrone in 100 mL 76% sulphuric acid) was added to the samples and the reaction mixture was heated at 40°C for 10–15 min. OD was taken at 620 nm using a spectrophotometer. A standard curve was drawn with different grades of pure sucrose dissolved in 0.1 mL of 30% KOH.

Total soluble sugars were determined by taking 0.05 mL from 80% ethanol extract (same as sucrose extract), mixed with 3 mL of anthrone reagent and kept in water bath at 90°C for 15 min, OD was taken at 620 nm. A standard curve was made by taking OD of different grades of glucose at the same wavelength.

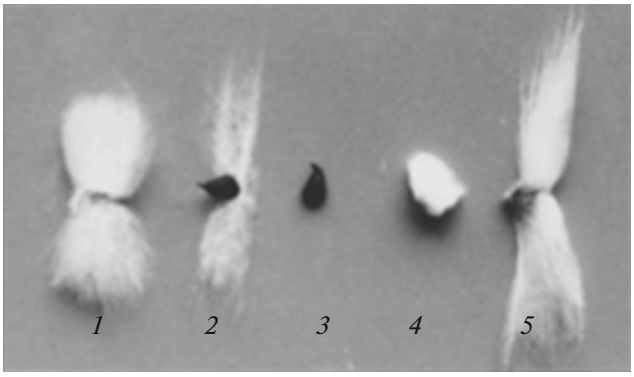

Fig. 1. Morphological overview of the mature seeds of cotton lines used in the experiment. The ovule or immature seed of these lines from –2 to 5 DPA were used for analysis. *1*—Xuzhou 142 Normal (wild-type, upland cotton) used as a control, *2*—Xuzhou 142-N (naked seeded) fuzzless mutant, *3*—Xuzhou 142-fl (fuzzless-lintless), *4*—Ligon lintless (Li), *5*—Sea Island PimaS-4 cotton.

Determination of INV activity. Preparation of crude enzyme extract: fresh cotton ovules (1 g) and separated fibers from 10 DPA ovules were crushed separately in 5 mL extraction buffer (0.1 M phosphate-citrate buffer pH 5.0) at 0°C. Extraction was carried out in an ice bath for 30 min. The slurry was centrifuged at 15000 *g* for 20 min at 4°C. The supernatant was collected; ammonium sulfate was added to 70% saturation; the mixture was placed in an ice bath for several hours. It was then centrifuged at 13000 *g* for 20 min at 4°C, and the precipitate was collected and dissolved in 0.3 mL extraction buffer followed by dialysis performed in the same buffer to remove ammonium sulfate and to get 1 mL final volume (crude enzyme extract).

Enzyme activity: sucrose was used as a substrate for sucrase/invertase and 1 mL of the reaction solution (0.25 mL citric acid pH 5.0, 0.2 M phosphate buffer mixed together, 0.1 mL 1 M sugar, 0.1 mL enzyme extract with 0.55 mL distilled water) was prepared. It was placed at 30°C in water bath for 60 min. DNS solution (1 mL) was added (6.3 g DNS (3,5-dinitrosalicylic acid), 2 g of 262 mL equivalent NaOH potas-

Table 1. Phenotypic characteristics of fiber and fuzz of five cotton materials

No.	Mutant name	Fiber (lint)	Short fiber (fuzz)
1	Xuzhou 142 Normal (wild type)	Normal	Many and densified
2	Xuzhou 142-N (naked seeded)	Normal	No
3	Xuzhou 142-fl (fuzzless-lintless)	No	No
4	Ligon lintless (Li)	Very short	Many and densified
5	Sea Island cotton (PimaS-4)	Normal and longer	A small amount

sium tartrate and sodium injection (dissolved 182 g in distilled water volume up to 500 mL) and the solution was heated, 5 g of the boiled extract was added to it along with 5 g anhydrous sodium sulfite. DNS solution was then heated, cooled and water was added to make total volume up to 1000 mL. It was kept in a dark place. To end the reaction the samples were heated for 5 min in a boiling-water bath, transferred immediately in cold water to cool reaction buffer and the absorption values were determined at 540 nm. The inactivated enzyme solution was added in advance to the above experimental mixture for use as a control. Glucose (20 ~ 100 μg) and 1 mL DNS solution were taken, heated for 5 min and OD was taken at 540 nm by using a spectrophotometer. A standard curve was drawn.

Determination of malic acid contents. Malic acid was extracted by weighing 0.5 g of cotton ovules preserved at −70°C and in which 0.6 N perchlorate solution was added 4 times the volume of plant material. It was then subjected to grinding. The slurry was centrifuged at 3000 *g* for 5 min at 4°C and the supernatant was transferred to ice cold tube, 0.02 mL (per 8 mL of supernatant) of methyl orange indicator (dissolve 50 mg in water with final volume 100 mL) was added and mixed. Added 5 M K_2CO_3 and placed on ice until CO_2 gas disappeared, added K_2CO_3 (pH 3.5) until color pink was attained. The mixture was then kept on ice for 10 min and then analyzed.

Malic acid contents: glycine amino acid compounds (0.4 M compounds, 0.5 M glycine, and pH 9.0) 2.5 mL; NAD solution (40 mM β-NAD) were mixed together and 0.2 mL of extraction buffer were added to this mixture. OD was taken for E1 at 340 nm, then 0.02 mL malic dehydrogenase (enzyme activity units of 34 μg/mL = 37 U/mg) was added to it. It was then heated at 37°C in a warm water bath for 30 min. OD for E2 was taken at 340 nm. Malic acid content calculated as C (mg/mL) = $(E_2 - E_1) \times 0.314F$ (where F is dilution factor).

RESULTS

Biochemical Changes in Cotton Ovules at Different Times of Flowering

Plant trichome development is associated with the appearance of spherical protrusions from the epidermal cells. Cotton fiber initiation represents a typical example of trichome development that originates as a protuberance from ovule epidermal cells at the time of anthesis. SuSy activity generates hexoses that act as the most important signal for initiation and as an integral part of cellulose biosynthesis during this cellular process [4, 10, 11]. The critical role of SuSy in the early stages of fiber initiation (0, −1 and 5 DPA) was investigated by using a fuzzless-lintless (fl) mutant with normal WT, N, Li and PimaS-4 cotton. The fl mutant failed to initiate fiber, leading to a fibreless appearance. The fluctuations in enzyme activities and sugar levels during fiber initiation to elongation can be coupled to changes in seed phenotype and fiber development.

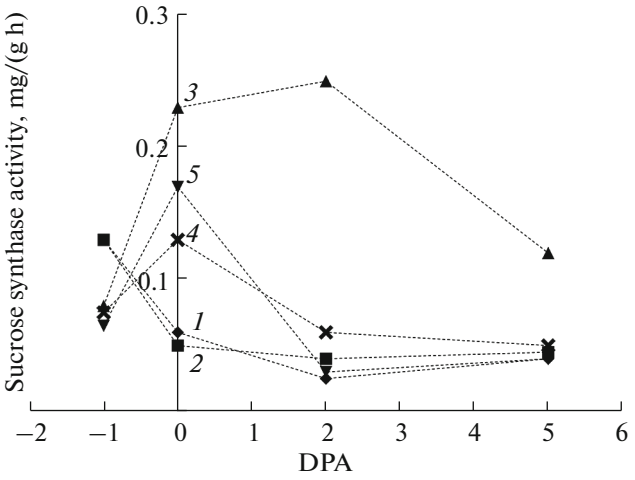

Fig. 2. SuSy activity in five different cotton materials from −1 to 5 DPA. *1*—Xuzhou 142-fl (fuzzless-lintless), *2*—Xuzhou 142-N (naked seeded), *3*—Xuzhou 142 Normal (wild-type, upland cotton), *4*—Ligon lintless (Li), *5*—Sea Island PimaS-4 cotton.

Trend in SuSy Activity, Sucrose and Total Soluble Sugar Contents from Pre-Anthesis to Post-Anthesis Day

SuSy activity. SuSy activity trend in isogenic lines fl and N was higher than in WT, Li and PimaS-4 day before flowering (−1 DPA), and then dropped substantially. In WT, SuSy activity on the flowering day reached the peak and then declined but was still high compared to the other four materials. The trend in Li and PimaS-4 within five days of flowering remained parallel with WT. Compared with −1 DPA, the 0 DPA SuSy activity in fl and N mutant ovules was 64.7 and 51.4% while in WT, Li and PimaS-4 it was 169.05, 68.11, and 154.84%, respectively (Fig. 2). The enzyme activity is in close agreement with large protrusions of fiber cell processes. Ovules bearing a large number of fibers initials exhibited significantly higher SuSy activity compared to N and fl mutant ovules with few or no cell projections on the flowering day. As the fiber cell elongation proceeds, SuSy activity decreases, which is not in close agreement with the initial elongation phase of fibers that is characterized by increased sugar levels. Therefore, it can be inferred that the increase in total soluble sugar contents in ovules is not caused by changes in the SuSy activity. Another possibility may be the presence of INV activity at the beginning of cell elongation.

Sucrose contents. Sucrose contents at −1 DPA in isogenic lines with same genetic background was in the following order: WT > N > fl mutant. In Li and PimaS-4, sucrose contents were low, which might be

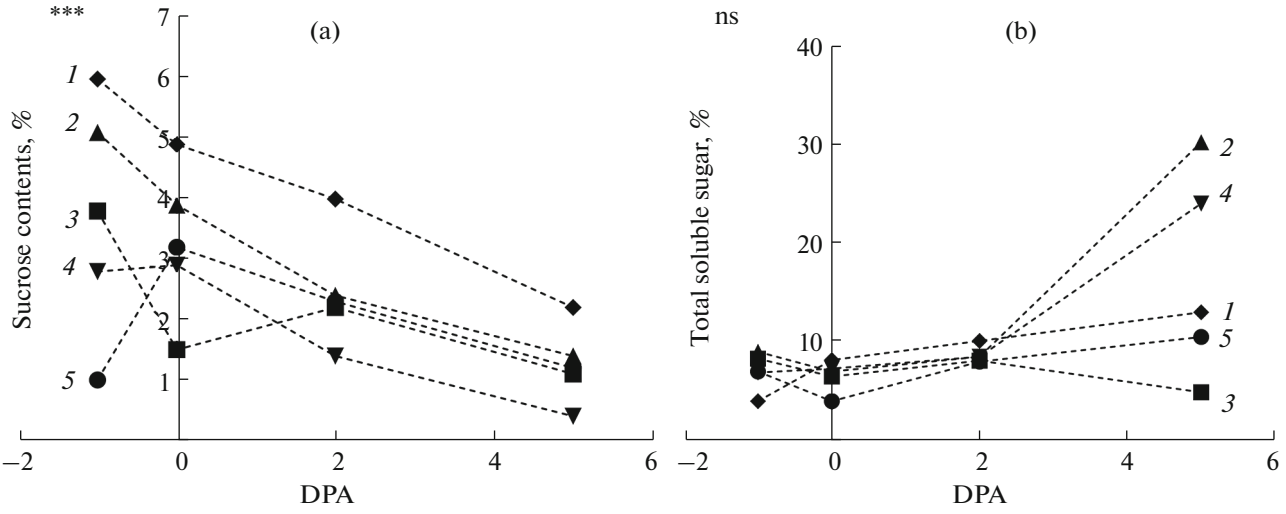

Fig. 3. (a) Sucrose contents analysis in five different cotton materials from −1 to 5 DPA. *1*—Ligon lintless (Li), *2*—Xuzhou 142 Normal (wild-type, upland cotton), *3*—Xuzhou 142-N (naked seeded), *4*—Sea Island PimaS-4 cotton, *5*—Xuzhou 142-fl (fuzzless-lintless). (b) Total soluble sugar contents analysis in five cotton materials from −1 to 5 DPA. *1*—Xuzhou 142 Normal (wild-type, upland cotton), *2*—Xuzhou 142-N (naked seeded), *3*—Ligon lintless (Li), *4*—Xuzhou 142-fl (fuzzless-lintless), *5*—Sea Island PimaS-4 cotton. Turkey test was used to compare the mean values; * indicate significant at $P = 0.05$, ** 0.01, *** 0.001 level of probability, while ns indicate non-significant.

related to the differences in the species. On 0 DPA, there was a change in the sucrose contents of five types of ovules: as PimaS-4 > WT > fl > Li > N mutants. In comparison with −1 DPA, the N mutant suffered the largest reduction in sucrose content (59.03%), followed by WT (22.83%) and PimaS-4 (16.17%). There was a significant increase (227.79%) in sucrose contents in fl mutants. Overall, PimaS-4, Li, WT and N ovules showed a decrease in sucrose content within 5 DPA. PimaS-4 and WT cotton ovules exhibited a sharp decline in sucrose contents than the other three materials. Sucrose content peaked on the same flowering day in the fl mutant. The results show that the differentiation of ovule epidermal cells into fiber cells requires a lot of sucrose reserves, especially during the initial stages of fiber formation. Sugars are gradually broken down by the enzymes for fiber elongation when needed, thus the sucrose contents are decreased (Fig. 3). The increase of sucrose contents in the fl mutant at 0 DPA may be caused by the changes in SuSy activity in embryo cells. As fertilization occurs on the flowering day (0 DPA), there is a need for a lot of energy to form an eight-cell nutrient-rich "embryo sac" which provides nourishment to developing embryos. Therefore, the ovules exhibit high SuSy activity as they require more nutrients in the form of carbohydrates. Sucrose is also used for seed growth; in the case of the fl mutant, seed size and ovule weight were large compared to the other four ovules used in the experiment. Therefore, fl mutant ovules showed elevated sucrose and SuSy activity at 0 DPA.

Total soluble sugar contents. Sucrose is the main translocated sugar in plants via phloem and considered the chief source of carbon supplied to fiber. During fiber growth sucrose is converted into other hexose sugars [23, 24]. In WT total soluble sugar contents were raised within five days of flowering with a slow increase up to two days of flowering and rapid increase later on. Consistency in soluble sugar changes was observed in WT, PimaS-4 and Li ovules before the flowering day. Comparison of total soluble sugar contents from −1 to 5 DPA showed an increase in PimaS-4 WT by 214.93 and 254.08%, respectively. While 200.07% increase in Li cotton and in fl and N mutants sugar contents remained low as compared to WT. From −1 to 5 DPA total soluble sugars increased 37.27 and 35.18% in N and fl mutant (Table 2).

From Fig. 3, it is evident that during −1 to 0 DPA no prominent difference was found in total soluble sugar contents between fl and four others but at 5 DPA there was a remarkable difference. The fiber stage of a cell has little effect on total soluble sugar contents while initial elongation at polarity phase requires a lot of sugars. Embryos and epidermal cells in process of growth and development require sugars but there is no surge of sugars during cell division and growth.

INV Activity in Five Seed Materials at 5 DPA

After repeating the experiment three times, it was found that intracellular invertase (INV) activity was not detected in −1 DPA ovules of all mutants and their wild types. However, fiber separated from 10 DPA ovules had a higher INV activity. On the other side, INV activity was not only absent in 10 DPA seeds (with fibers) of WT but also in 10 DPA seeds (without fibers)

Table 2. Total soluble sugar contents in five cotton ovules from −1 to 5 DPA

Flower days		Material			
The day before (−1 DPA)	X_i–Y_j	2	3	4	5
	1	3.0363*	0.8876	−1.8569*	0.6123
	2		−2.1487*	−4.8932*	−2.424*
	3			−2.7445*	−0.2754
	4				2.4692*
Flowering day (0 DPA)	1	−2.3523*	−0.6695	−0.5193	1.4656
	2		1.6828	1.833	3.8179*
	3			0.1502	2.1351
	4				1.9849
For two days	1	−1.3415	0.06188	0.2041	0.5274
	2		1.4034*	1.5456*	1.8689*
	3			0.1422	0.4656
	4				0.3233
Five days	1	−2.9471	20.3944*	−5.3033*	15.3312*
	2		23.3415*	−2.3561	18.2783*
	3			−25.6976*	−5.0632*
	4				20.6345*

* Indicate significant at the $P = 0.05$ level of probability. Total soluble sugar content (mg/mL): X_i – plant material; Y_j – DPAs.

of fl mutant. Therefore, we can speculate that there may be some inhibitory substances for INV expression in seed epidermal cells which could be transferred into fiber cells. Separated fibers, however, cannot get these inhibitory substances from ovule epidermal cells and have a higher INV activity. This mechanism is not clear yet and needs to be explored.

Inhibition of INV may lead to blockage of fiber initiation. The results obtained coincide with earlier findings in which silencing of GHVIN1 through RNAi obstructed fiber initiation from ovule epidermis [24]. In previous studies, GhCWINV1 mRNA was found in dividing cells of endosperm but absent in discrete cells that showed its role in the nuclear division but not in the synthesis of the cell wall in endospermic cells [25]. Increasing CWINV expression by down-regulation of its inhibitors increased seed weight in tomato and soybean [25–27].

Malic Acid Contents

Malic acid contents changes within isogenic lines WT and fl mutants at −1, 0 and 3 DPA were not significant. In the context of non-varietal differences, no significant variation was observed. Therefore, malic acid is not critical in cell projection. However, difference reached a considerable level at 5 DPA when WT cotton fiber cells entered into the initial development stage (Table 3). Malic acid might have an influence on the fiber cell elongation. After spending two days in flowering no significant difference in malic acid was observed in PimaS-4 and WT cotton. The significant difference within specified time period was observed in other lines. Malic acid contents were conflicting with WT and PimaS-4 at −1, 0, 5 DPA that could possibly be associated with increased rate of cell elongation. Therefore, total soluble sugars, INV, and malic acid showed no striking effect on fiber initiation but seemingly play their role in fiber elongation. Earlier investigations found a positive relationship between malic acid and high vacuolar invertase activity [28].

DISCUSSION

The study shows that the fate of ovule epidermal cells to bulge out and differentiate into fibers is coupled to SuSy activity. A significant decrease in activity at 0 DPA results in fibreless or only a few shrunken and rudimentary fuzz-like fibers on the naked seed surface. In the case of the fl mutant, no fiber appeared which might be due to a decrease in SuSy activity at the flowering day. The elevated level of sucrose in fl mutant ovules is due to a lack of fiber cell initiation on the ovule epidermis, a decrease in SuSy activity and altered carbon partitioning [4, 5, 7].

At 0 DPA, WT (wild-type) cotton ovule epidermal cells have a large number of fiber initials; N mutant (Xuzhou 142-N naked seed, fuzzless) only has a few

Table 3. Analysis of malate contents (mg/mL) of five cotton ovules from −1 to 5 DPA

Materials	Flower days			
	The day before (−1 DPA)	Bloom day (0 DPA)	Three days (3 DPA)	Five days (5 DPA)
PimaS-4	5.76 ± 0.04	5.66 ± 0.09	5.15 ± 0.46	5.98 ± 0.08
Li	6.34 ± 0.16	6.27 ± 0.13	5.46 ± 0.12	4.98 ± 0.11
fl	6.6 ± 0.22	6.89 ± 0.07	5.44 ± 0.1	5.94 ± 0.37
WT	7.34 ± 0.58	6.63 ± 0.2	4.73 ± 0.11	4.68 ± 0.04

Each value is mean ± SE of three replicates.

fiber initials on epidermal cells at the chalazal end, while the fl mutant (Xuzhou 142-fl fuzzless-lintless) showed no significant cytoplasmic protuberances. SuSy activity in WT, N and fl mutant ovules was highest before flowering and declined sharply on the flowering day, reaching the lowest point two days after flowering. The number of fiber initials on ovule epidermal cells of three isogenic lines is in agreement with SuSy function. The SuSy activity of PimaS-4 on each flowering day was much higher than on the day before flowering due to the consumption of a lot of sugars. The number of fiber cells in mutant ovules greatly reduced, with only a few fiber cells on the chalazal side when compared to the normal control. Therefore, changes in SuSy activity trend are in agreement with fl mutants. Ligon cotton is characterized by a very slow elongation rate on the 5th day compared to Li, which has the same rate as WT before the 5th day. On the flowering day, ovule epidermal cells have uniform fiber protuberances which are in accordance with phenotypic appearance of the normal wild-type. PimaS-4 fiber cell processes are in conformity with WT, but the number of fiber initials might vary between different cotton lines, leading to variations in SuSy activity. In short, the SuSy activity level in ovules was directly related to the rate of fiber cell projections on each flowering day. Therefore, SuSy is important for initiation when fiber cell protrusions require a large amount of sucrose, but the relationship with the changes in total soluble sugar content were not in accordance with the expansion of fiber cells. The results coincide with a previous study showing that decreased SuSy activity represses trichome development, elongation and seed development. Cellulose-rich fibers were not formed when SuSy activity decreased significantly [21].

The increase in total soluble sugars and the decrease in SuSy in WT, Li, and PimaS-4 during the initial stages of fiber development, especially after the 5 DPA, indicate that the metabolism shifts from SuSy to INV. Previous studies reported that SuSy gene RNA expression was greatly reduced in ovule epidermal cells of fibreless seed mutant (fls) on anthesis day (0 DPA), whereas SuSy proteins were at a significant level in wild-type seed (FLS) ovule epidermal cells [5]. In the mutant ovule epidermis, bud-like projections were not observed at 0 DPA, whereas these projections were abundant in wild-type ovules. The data support our finding that there is a strong association between SuSy and fiber development. The increased SuSy may be due to the fact that this enzyme catalyzes the cleavage of sucrose into glucose and fructose, which is evident from an increase in total soluble sugars and a decrease in sucrose contents. High sucrose contents in PimaS-4 ovules indicate that sucrose breakdown decreased due to the low SuSy activity. When elongation starts, decrease in sucrose results in increase in total soluble sugars. It can be hypothesized that the normal expression of SuSy in the ovule on the flowering day provides energy and biosynthetic precursors for cell expansion. On the other hand, the expression of SuSy, which facilitates fiber cell formation, might be associated with the need for fiber cell turgor pressure. Rapid cell expansion is associated with high turgor within the cell which is sustained by hexoses generated by SuSy activity in cotton seeds [1, 7].

Maintenance of turgor pressure in fiber cells is due to soluble sugars, K^+, and malic acid contents. The study showed that differences in total soluble sugars and malic acid contents within WT and N ovules are not prominent. However, there is a significant difference in total soluble sugars and malic acid content in *Gossypium hirsutum* and *G. barbadence* that might be due to varietal differences. Although the ovule intracellular K^+ concentration was not assessed, the proportion of total soluble sugars in WT, N, Li and PimaS-4 cotton ovules on the flowering day was over 5%, which reflects that soluble sugars should presumably have a great impact on turgor pressure. Therefore, turgor pressure of ovule epidermal cells and the number of fiber initials are not closely related. However, within 5 DPA, the ovule results are reversed. Total soluble sugars and malic acid contents in WT were significantly higher compared to those in N and fl mutants. At 5 DPA, fibers entered the rapid elongation phase, suggesting that the early elongation of cells requires a lot of soluble sugars and malic acid, which may be related to maintaining high turgor within the cell [22, 29]. Most of the studies favor the osmotic pressure regulation theory in cases of fiber cell elongation [1]. From the current study, it is clear that turgor pressure is important for elongation rather than initiation. The initial protrusion of fibers may be due to cell wall extensibil-

ity instead of turgor pressure, as reported by previous studies [4].

In plant cells, the breakdown of sucrose takes place by the SuSy and INV enzymes. INV perhaps remains silent during early fiber initiation as INV activity was not detected in cotton ovules, but it was very high in elongating fiber cells. Therefore, it can be speculated that the enzyme is present in epidermal cells but that embryo or seed coat cells inhibit its activity, so it could not be detected in the cotton ovules. In fl mutant, changes in ovule's sucrose contents do not result from INV but rather due to SuSy activity. It is evident from the experiment that in fl mutant no considerable epidermal cell protrusions were observed so demand for sugars is not very high. The study revealed that decrease in SuSy activity on the flowering day (0 DPA) led to an increase in the sucrose content and decrease in total soluble sugars. However, during the initial fiber development stage, especially at 5 DPA, the total soluble sugar content within ovules was greatly increased and sucrose content was reduced. This change was caused by INV because there was a decline in SuSy activity trend on the flowering day which might be caused by INV, although no change in activity was detected.

Overall, in this experiment, the fibreless mutant was used to verify the role of INV in promoting the emergence of fiber cell initials. A close relationship exists between turgor pressure and fiber elongation rather than initiation, as reported previously [19]. The results of the current study are in accordance with previous findings [22].

The above study illustrates the role of SuSy, INV, sucrose and total soluble sugars in the mechanism of fiber initiation from cotton ovule epidermal primordia. Fiber cell initiation and SuSy activity are coupled to each other. Changes in metabolite levels and the enzymatic activities of SuSy and INV have a strong correlation with structural alterations in seeds and the seed phenotype. In addition to the above-mentioned constituents, there are many genes, transcription factors, and plant hormones that interact with the process of fiber initiation and development. The fl mutant ovules will provide an opportunity to examine these aspects at a molecular and genetic level. Thus, understanding the role of genes and hormones will eventually lead to an increase in fiber yield and quality through molecular breeding and stable genetic transformation.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China and Zhejiang Province Science Research Project (project nos. 31671763, 31471567 and 2017C32077).

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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