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Changes in sugar composition and cellulose content during the secondary cell wall biogenesis in cotton fibers

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Abstract Two cotton cultivars TX19 and TX55 (*Gossypium hirsutum* L. cv.) were planted in the greenhouse and fibers were harvested at different stages of development. The percentage of sugars present on the fibers was determined by High Performance Liquid Chromatography and the cellulose content was determined using the anthrone method. The percentage of sugars (sucrose, glucose, fructose, and galacturonic acid) showed statistically significant changes during fiber development. The decrease in the percentages of these sugars as the secondary cell wall develops was associated with an increase in the cellulose content. It is important to point out that these analyses were done on intact fibers, no cell wall extractions and purifications were performed.

Keywords Cotton · Fiber development · Gossypium · Secondary cell wall · Cellulose

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Introduction

Cotton fiber is composed of 95% cellulose (1–4 linked β-D-glucose). Several overlapping developmental phases lead to the formation of mature cotton fibers: initiation, elongation, secondary cell wall deposition, and finally maturation (Seagull et al. 2000; Jasdanwala et al. 1977; Kim and Triplett 2001; Wang et al. 2009). The day of flowering is referred to as anthesis and the term "days post-anthesis" (dpa) is often used to describe the cotton fiber development. Fiber initiation, which commences at 0 dpa, signals the onset of fiber morphogenesis. Fiber growth is characterized by the synthesis of the primary cell wall and an increase in fiber length up to $\sim$30 mm within 3 weeks after anthesis. The stage of secondary cell wall development commences in general around 21 dpa and continues for a period of $\sim$3 to 6 weeks post-anthesis. This phase is marked by a massive deposition of a thick cellulosic wall (Wilkins and Jernstedt 1999). The transition period between 16 and 21 dpa is considered to represent a developmental switch in emphasis from primary to secondary cell synthesis during cotton fiber development. Cotton fiber secondary cell wall is almost pure cellulose (Haigler et al. 2005). While the primary cell walls of fibers have been reported to contain between 35 and 50% cellulose, the remaining portion is composed of different polysaccharides (Huwyler et al. 1979; Tokumoto et al. 2002). Several studies have been conducted to determine the composition of the

polysaccharides matrix in developing cotton fibers (Meinert and Delmer 1977; Huwyler et al. 1979; Tokumoto et al. 2002; Maltby et al. 1979; Timpa and Triplett 1993). The results showed that acidic polymers (galacturonans), β-glucans (β-1,3-glucans), and xyloglucans showed significant quantitative changes during cotton fiber development. These experiments were performed on extracted polysaccharides matrix from cell wall through a series of fractionation.

Timpa and Triplett (1993) reported on the analysis of cell-wall polymers during cotton fiber development (*Gossypium hirsutum L.*). Using Gel Permeation Chromatography, cell-wall polymers from cotton fibers at different developmental stages were analyzed. The authors showed that at the primary cell wall development stage, cell wall polymers had on average lower molecular weights than those from fibers at the secondary cell wall development stage. However, cellulose macromolecules with high molecular weight, that are characteristic of mature cotton, were detected as early as 8 dpa (days post anthesis). In another study, purified cell walls prepared from cotton fibers (*Gossypium arboreum L.*) at different growth stages, were subjected to successive extractions to establish pectic, hemicellulosic, and cellulose fractions (Huwyler et al. 1979). The results showed that the absolute amounts of fucose, galactose, manose, rahmnose, arabinose, uronic acid, and non-cellulosic glucose residues all reached a maximum at the end of the primary cell wall formation or at the beginning of the secondary wall formation (Huwyler et al. 1979).

It has been suggested that, although some synthesis of cellulose was possible from supplied UDP-Glc (uridine diphosphate-glucose) in vitro, cellulose synthesis occurs more effectively if sucrose, rather than UDP-Glc, was the supplied substrate (Delmer 1999). During the growth, sucrose is transformed by enzymes into glucose and fructose. Then enzymes convert fructose to glucose and polymerization reactions between glucose units lead to the formation of cellulose macromolecules.

In previous research, we reported on the study of fiber development using Fourier Transform Infrared spectroscopy (FTIR) and Thermogravimetric Analysis (TGA) (Abidi et al. 2008, 2009). Our results indicated that these 2 analytical techniques could provide useful information related to structural changes that occur during cotton fiber development. Specifically, it was possible to determine precisely the transition phase between primary cell wall synthesis and secondary cell wall synthesis. It is important to point out that all FTIR measurements and TGA tests were performed on intact cotton fibers at different stages of development. No extractions of cell wall components were performed. Studying fiber development using intact fibers present two major advantages: (1) time consuming cell wall multiple extractions and purifications are avoided, and (2) the integrity of the cell wall structure is preserved allowing to study its composition and variability by means of FTIR imaging techniques for example.

In this paper, we report on the changes in the sugar composition of cotton fibers as determined by High Performance Liquid Chromatography, and the cellulose content as determined by the anthrone method during the secondary cell wall biogenesis.

Materials and methods

Cotton fibers

For this study, 2 replications (10 plants each) of two cotton cultivars (*Gossypium hirsutum* L. cv. TX19 and TX55) were planted in a greenhouse with day/night cycles varying from 13/11 to 11/13 h and day/night temperatures of about 31 °C/24 °C. Plants were grown in 20 L (5 gallons) pots of Sungrow SB 300 potting mix that had been amended with Peters 15-9-12 slow release fertilizer prior to potting. Plants were watered as needed. On the day of flowering (0 dpa), individual flowers were tagged, and 14 developing bolls per cultivar and per replication were harvested at 10, 14, 17, 18, 19, 20, 21, 24, 27, 30, 36, 46, and 56 dpa. The pericarp was immediately removed (excised with scalpel) and isolated ovules were transferred to cryogenic vials and stored in a Cryobiological Storage System filled with liquid nitrogen until analyses were performed. Each replication was tested independently.

High performance liquid chromatography (HPLC)

Sample preparation for HPLC analysis was done as followed: for fibers from TX55 cultivar from 10 to 21 dpa and for fibers from TX19 cultivar from 10 dpa to 17 dpa, fibers were separated from the seed and

dried in a Petri dish for 2 days at 40 °C. Then they were weighed and placed in glass vials (8 mL). About 4 mL of HPLC water (18.2 megohm water) were added in the vials and the solutions were homogenized using Homogenizer Pro200 with 7 mm generator at 12000–17000 rpm (PRO Scientific, CT). The solutions were transferred to a 25 mL volumetric flask and the volume was adjusted with HPLC water. A sample of the aqueous solution was taken from the flask with a 10 cm^3 syringe on which a 0.2 micron filter (nylon membrane-polypropylene housing) (National Scientific, Scottsdale, AZ) was attached. A 1.5 mL filtered sample was deposited into a 1.5 mL autosampler vial (C4013-15A, National Scientific). Sugars were separated on the columns (CarboPac PA1 Anion exchange Guard column and two CarboPac PA1 Anion exchange Analytical Columns [Dionex Corporation, Sunnyvale, CA]) in series with a Gradient Eluent system: Eluent 1: 200 mM NaOH and Eluent 2: 500 mM Sodium Acetate ($C_3H_3Na_2$ $3H_2O$) and 200 mM NaOH. The following sugars commonly found in the plant cell wall were analyzed by HPLC: glucose, sucrose, fructose, fucose, rhamnose, arabinose, galactose, xylose, mannose, galacturonic acid, glucuronic acid (Tokumoto et al. 2002; Huwyler et al. 1979).

For fiber samples from TX55 cultivar (24 to 30 dpa) and for fibers from TX 19 cultivar (18 to 30 dpa), fibers were separated from the seed and dried in a Petri dish for 2 days at 40 °C. Then, they were grinded in a Wiley Mill to pass 20 mesh, placed into a plastic bag and 25 mL of HPLC water were added. The plastic bag was placed in the stomacher for homogenization. A sample of the aqueous solution was taken from the bag with a 10 cm^3 syringe on which a 0.2 micron filter (nylon membrane-polypropylene housing) was attached and the HPLC tests were performed as indicated above. For fiber samples from TX 55 cultivar (36 to 56 dpa) and for fibers from TX19 cultivar (36 dpa to 56 dpa), fibers were ginned and dried at 40 °C for 2 days. Then, the same procedure was repeated as with samples from 24 to 30 dpa.

Sample dehydration for cellulose content determination

An established dehydration procedure for frozen samples was carried out as previously described (Abidi et al. 2008; Muller and Jacks 1975; Rajasekaran et al. 2006). The dehydration procedure consisted of washing the hydrated sample (previously rinsed with water) with acidified 2,2-dimethoxypropane (one drop of HCl in 50 mL of 2,2-dimethoxypropane), followed by five exchanges for 15 min each in 100% acetone. In a slightly acidic solution, 2,2-dimethoxypropane is instantly hydrolyzed by water to form methanol and acetone (Muller and Jacks 1975).

Cellulose content determination

Cellulose content of developing cotton fibers was determined using the anthrone method (Viles and Silverman 1949). The anthrone, a tricyclic hydrocarbon ($C_{14}H_{10}O$), is generally used for cellulose assay and colorimetric determination of carbohydrates. This method consisted of adding anthrone solution (0.05 to 0.20%) in concentrated sulfuric acid to an aqueous solution of cotton fibers (previously digested by sulfuric acid). The absorbance of the green color of the solution is measured using a UV–Vis spectrophotometer LAMDA650 (PerkinElmer, USA) at 625 nm and it is proportional to the cellulose content of the sample. Microcrystalline cellulose (Avicel) was used as a standard for the calibration.

Results and discussion

It is generally accepted that sucrose, the major translocated sugar via the phloem tissues is the main source of carbon supplied to the fibers (Tarczynski et al. 1992; Delmer 1999). During the growth and development of cotton fibers, sucrose is converted by enzymes to glucose and fructose. Then, fructose is converted by enzymes to glucose. This is followed by polymerization reactions of glucose units to form cellulose macromolecules. Consequently, all the intermediate compounds produced during the biosynthesis of cellulose from sucrose may be found on cotton fibers or within the lumen.

The evolution of the percentage of sucrose as function of dpa for both cultivars is exhibited in Fig. 1. The percentage of sucrose is much lower than the percentage of glucose and fructose. There is a statistically significant effect of the developmental stage but no effect of cultivars on the percentage of

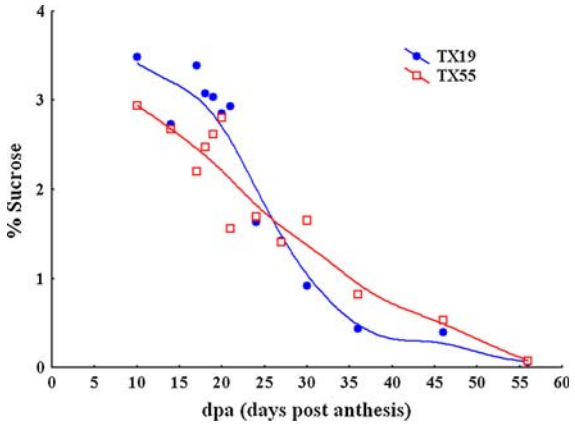

Fig. 1 Evolution of the percentage of sucrose as function of developmental stage (the percentage is expressed in percent of the weight of the fiber)

sucrose nor there is interaction cultivar*dpa (Table 1).

Figure 2 shows the evolution of the percentage of glucose as a function of developmental stages (dpa) for both TX19 and TX55 cultivars. The statistical analysis (analysis of variance) shows significant effects of both cultivars and developmental stage on the percentage of glucose (Table 2). For fibers from TX19 cultivar, the percentage of glucose at 10 dpa is 27.3% (expressed on the weight of the fiber). This percentage decreases as the fiber develops and at 36 dpa it is around 1.2%. For fibers from TX55 cultivar, the percentage of glucose is much higher at 10 dpa (39.1%) and starts decreasing beginning at 21 dpa. Statistically significant interaction cultivar * dpa is observed. Overall, between 10 dpa and 36 dpa the percentage of glucose in fibers from TX19 cultivar is lower than in fibers from TX55 cultivar.

Figure 3 shows the evolution of the percentage of fructose as a function of the developmental stage for both cultivars. A gradual decrease is observed for both cultivars between 10 dpa and 36 dpa. There is statistically significant effects of both cultivar and the development stage on the fructose content but no statistically significant interactions cultivar*dpa (Table 3).

Figure 4 and Table 4 show the evolution of the percent of galacturonic acid for both cultivars. During the early stage of development (between 10 dpa and 18 dpa), fibers from TX19 and TX55 cultivars exhibited significant differences. Fibers from TX19 cultivar exhibited higher percentages of galacturonic acid than fibers from TX55 cultivar at 10, 14, and 17 dpa. This percentage decreased beginning at 20 dpa and leveled of at 0% at 36 dpa.

Table 1 Variance analysis: effect of developmental stage (days post-anthesis) and cultivars on the percentage of sucrose (expressed in percent of the weight of the fiber)

Parameter	*df*	*F*	Probability	% Sucrose[a]
Intercept	1	551.0	0.000001	
Cultivar	1	1.9	0.181953	
dpa	12	12.9	0.000001	
10				3.2 a
14				2.7 a
17				2.8 ab
18				2.8 ab
19				2.8 ab
20				2.8 ab
21				2.3 abc
24				1.7 cd
27				1.4 cd
30				1.3 cd
36				0.9 de
46				0.5 de
56				0.1 e
Cultivar*dpa	12	1.0	0.465392	
Error	26			

df, degrees of freedom; *F*, variance ratio, [a]Values not followed by the same letter are significantly different with $\alpha = 5\%$ (according to Newman-Keuls tests)

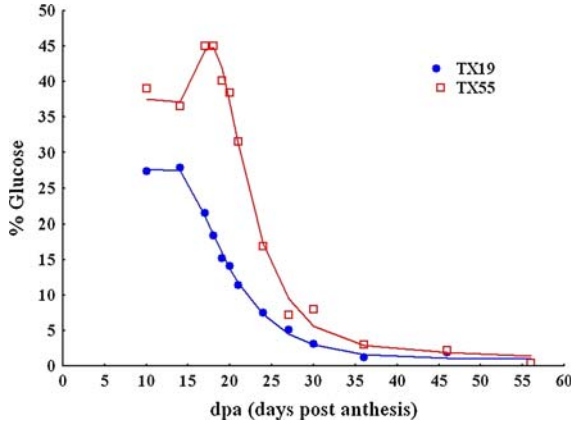

Fig. 2 Evolution of the percentage of glucose as function of developmental stage (the percentage is expressed in percent of the weight of the fiber)

Table 2 Variance analysis: effect of developmental stage (days post-anthesis) and cultivars on the percentage of glucose (expressed in percent of the weight of the fiber)

Parameter	*df*	*F*	Probability	% Glucose[a]	
				TX19	TX55
Intercept	1	714.3	0.000001		
Cultivar	1	82.5	0.000001		
dpa	12	30.8	0.000001		
10				27.3 bcd	39.1 ab
14				27.9 bcd	36.5 ab
17				21.5 cde	45.0 a
18				18.3 cdef	45.0 a
19				15.2 def	40.1 ab
20				14.1 def	38.4 ab
21				11.4 ef	31.5 abc
24				7.5 ef	17.0 cdef
27				5.1 ef	7.2 ef
30				3.1 f	8.0 ef
36				1.2 f	3.0 f
46				1.9 f	2.2 f
56				0.1 f	0.4 f
Cultivar*dpa	12	4.5	0.000649		
Error	26				

df, degrees of freedom; *F*, variance ratio, [a]Values not followed by the same letter are significantly different with $\alpha = 5\%$ (according to Newman-Keuls tests)

Table 3 Variance analysis: effect of developmental stage (days post-anthesis) and on the percentage of fructose (expressed in percent of the weight of the fiber)

Parameter	*df*	*F*	Probability	% Fructose[a]
Intercept	1	1127.1	0.000001	
Cultivar	1	34.7	0.000003	
dpa	12	48.9	0.000001	
10				30.3 a
14				27.6 a
17				21.3 b
18				20.8 b
19				20.5 b
20				19.5 b
21				14.1 c
24				9.7 d
27				6.3 de
30				5.3 de
36				1.5 e
46				1.4 e
56				0.3 e
Cultivar*dpa	12	1.8	0.104889	
Error	26			

df, degrees of freedom; *F*, variance ratio, [a]Values not followed by the same letter are significantly different with $\alpha = 5\%$ (according to Newman-Keuls tests)

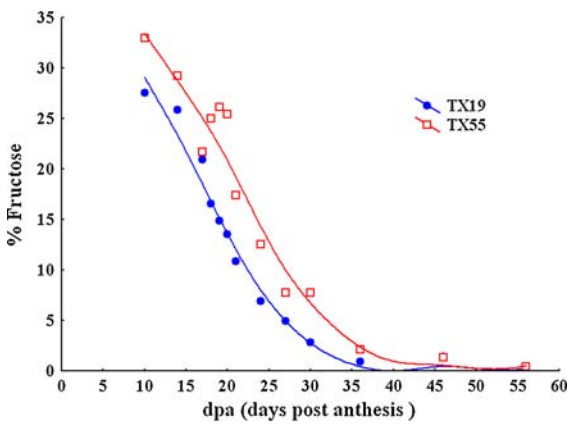

Fig. 3 Evolution of the percentage of fructose as function of developmental stage (the percentage is expressed in percent of the weight of the fiber)

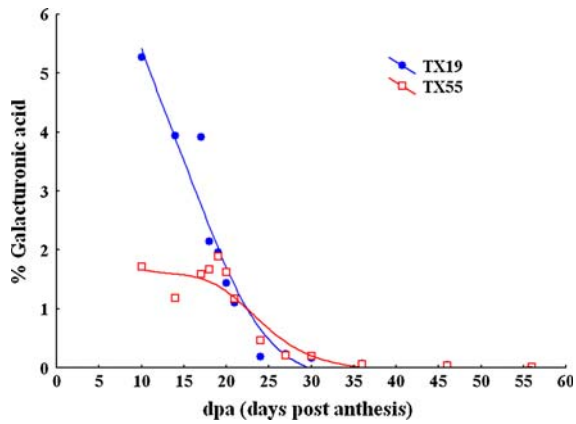

Fig. 4 Evolution of the percentage of galacturonic acid as function of developmental stage (the percentage is expressed in percent of the weight of the fiber)

Fucose, arabinose, galactose, mannose, and xylose were detected only as traces, while less than 0.3% of rhamnnose and glucuronic acid were detected.

Cellulose content was measured using the anthrone method. Microcrystalline cellulose (Avicel) was used as a standard for calibration of the UV–Vis

Table 4 Variance analysis: effect of developmental stage (days post-anthesis) and cultivars on the percentage of galacturonic acid (expressed in percent of the weight of the fiber)

Parameter	*df*	*F*	Probability	% Galacturonic acid[a]
Intercept	1	73.4	0.000001	
Cultivar	1	5.3	0.029838	
dpa	12	5.3	0.000181	
10				3.5 a
14				2.6 ab
17				2.8 a
18				1.9 abc
19				1.9 abc
20				1.5 abc
21				1.1 abc
24				0.3 bc
27				0.2 bc
30				0.2 bc
36				0.1 c
46				0.0 bc
56				0.0 bc
Cultivar*dpa	12	1.5	0.172426	
Error	26			

df, degrees of freedom; *F*, variance ratio, [a]Values not followed by the same letter are significantly different with $\alpha = 5\%$ (according to Newman-Keuls tests)

spectrophotometer. Figure 5 shows the evolution of the cellulose content as a function of dpa for both cultivars. The statistical analysis shows significant effects of both cultivar and developmental stage on the cellulose content (Table 5). There is also statistically significant interaction cultivar*dpa. For fibers from TX 19 cultivar, the cellulose content is around 10.3% at 10 dpa. There is no statistically significant change in the cellulose content between 10 dpa and 14 dpa. However, at 17 dpa the cellulose content jumps to 33.9% and then to 56.9% at 18 dpa. This drastic increase in the amount of cellulose is attributed to the initiation of the secondary cell wall synthesis around 17 dpa. The fast cellulose rate synthesis occurs between 14 dpa and 24 dpa. During this period, the cellulose content increases from 10.7 to 80.4%. Significant but slow increase of cellulose content is observed beyond 24 dpa. The final amount of cellulose in fibers from TX19 is 95.0%. These results are in agreement with previous results (Tokumoto et al. 2002). The authors reported that the amount of cellulose increased continuously during fiber development, and that the increase was very important at the end of the fiber elongation phase (beginning of the thickening stage).

For fibers from TX55 cultivar, there is no significant change of cellulose content between 10 dpa and 20 dpa. The average cellulose content is 9.1%. The cellulose content jumps to 34.1% at 21 dpa and to 68.3% at 24 dpa. Between 27 dpa and 56 dpa, there is no statistically significant difference in the cellulose content. The final amount of cellulose in fibers from TX55 at 56 dpa is around 88.4%.

The comparison of the cellulose content of fibers from TX19 and TX55 shows that the cellulose synthesis in fibers from TX19 starts as early as 18 dpa. However, in fibers from TX55 cultivar, the cellulose synthesis starts at 24 dpa. There is 6 days difference in the cellulose synthesis and, therefore, in the secondary cell wall initiation. This result is extremely important because a few days difference in the initiation of the secondary cell wall synthesis could have a large impact on fiber maturity at the end of the growing season. Indeed, at the end of maturation phase (56 dpa) cellulose content of fibers from TX19 is 95.0% while it is only 88.4% in fibers from TX55 cultivar. It is important to point out that fiber maturity is a major yield component and an important fiber quality trait that is directly linked to the quantity of cellulose during the secondary cell wall synthesis. Low maturity fibers tend to be weak and to break during processing (ginning, opening,

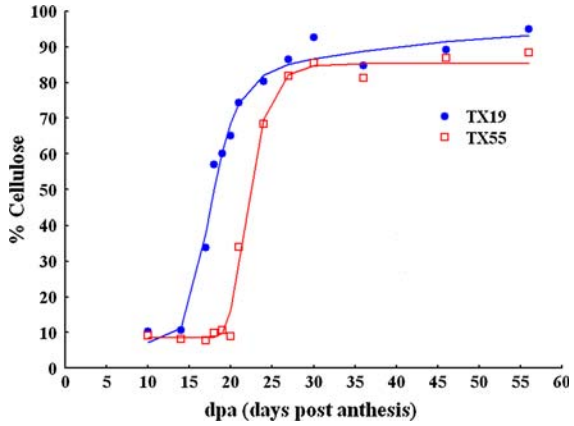

Fig. 5 Evolution of the cellulose content as function of developmental stage (dpa)

Table 5 Variance analysis: effect of developmental stage (days post-anthesis) and cultivar on the cellulose content

Parameter	*df*	*F*	Probability	% Cellulose[a]	
				TX19	TX55
Intercept	1	7786.5	0.000001		
Variety	1	258.9	0.000001		
dpa	12	202.1	0.000001		
10				10.3 f	9.1 f
14				10.7 f	8.1 f
17				33.9 e	7.8 f
18				56.9 d	9.8 f
19				60.1 d	10.6 f
20				65.1 cd	9.0 f
21				74.3 bc	34.0 e
24				80.4 ab	68.3 cd
27				86.5 ab	81.8 ab
30				92.6 a	85.6 ab
36				84.7 ab	81.3 ab
46				89.1 ab	86.8 ab
56				95.0 a	88.4 ab
Variety *dpa	12	21.9	0.000001		
Error	26				

df, degrees of freedom; *F*, variance ratio. [a]Values not followed by the same letter are significantly different with $\alpha = 5\%$ (according to Newman-Keuls tests)

carding, weaving). This results in poor yarn quality and ultimately leads to poor quality fabrics with lower dye affinity (appearance of white specks).

It is important to stress that although the percentages of sucrose, glucose, and fructose are lower in fibers from TX19 cultivar compared to fibers from TX55 cultivar at every developmental stages, the cellulose content in these fibers is higher compared to fibers from TX55 cultivar. This leads us to hypothesize that the enzymatic activities (biosynthesis of cellulose from sucrose) is more elevated in fibers from TX19 cultivar than in fibers from TX55 cultivar. This hypothesis needs to be confirmed.

Conclusion

High Performance Liquid Chromatography analysis of sugars content on fibers harvested at different stages of development shows statistically significant changes of the percentages of sucrose, glucose, fructose, and galacturonic acid. The percentages of these sugars decrease as the secondary cell wall develops. These sugars are essential for cellulose synthesis allowing the formation of the secondary cell wall. Indeed, the amount of cellulose content determined by the anthrone method increases as the fiber develops. All these analyses were performed on intact fibers. No cell wall extractions and purifications were performed.

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