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Effect of H_2O_2 on fiber initiation using fiber retardation initiation mutants in cotton (*Gossypium hirsutum*)

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ABSTRACT

Single-celled fibers initiate at anthesis from cotton seed epidermal cells of normal developmental cotton cultivars; however, fiber initiation is retarded in some cotton fiber mutants. In this study, the relationship between genes associated with fiber initiation retardation and fiber initiation development was investigated using three cotton fiber developmental mutants: recessive naked seed n2; dominant naked seed N1; and Xinxiangxiaoji Linted-Fuzzless Mutant (XinFLM); with genetic standard line TM-1 (TM-1) as control. Retardation during fiber initiation development was observed in N1 and XinFLM by scanning electron microscope (SEM) analysis. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of genes related to the fiber initiation development showed that the expression of *GhEXP1* and *GhMYB25* was lower in N1 and XinFLM than in TM-1 and n2, however, the expression of *GhTTG1* and *GhTTG3* in XinFLM and n2 was higher than in TM-1 and N1. *In vivo* and *in vitro* treatments on ovules demonstrated that 30% hydrogen peroxide (H_2O_2) could prevent fiber initiation retardation in XinFLM, but no evident effect on N1. To further confirm the relationship between gene expression and the effects of H_2O_2 in XinFLM, qRT-PCR analysis of four differentially expressed genes was performed using −1 d post-anthesis (DPA) ovules of XinFLM treated for 24 and 48 h with 30% H_2O_2 and H_2O, respectively, with 0 and 1 DPA untreated ovules from XinFLM and TM-1 as control. The results showed that the expression of *GhMYB25* and *GhEXP1* showed significant difference in XinFLM after −1 DPA ovule treated for 24 h relative to the untreated or H_2O-treated ovules, with the expression of *GhMYB25* increased significantly and that of *GhEXP1* decreased. This implied that H_2O_2 might be one of the upstream signal molecules affecting the expression of *GhMYB25* and *GhEXP1* genes. The fiber initiation retardation in XinFLM might be related to the production of reactive oxygen species (ROS).

Introduction

Cotton fiber development is divided into four overlapping growth phases: initiation, elongation (primary cell wall synthesis), secondary cell wall synthesis, and maturation (Willison and Brown, 1977; Basra and Malik, 1984; Ruser, 1985; Graves and Stewart, 1988). The emergence of the fiber initiation at the day of anthesis had been illustrated by scanning electron microscopy (SEM) analysis, which the balloon-like cells were visible through bulging and spherical expansion above the epidermal surface of cotton ovules and followed by cell elongation (Beasley, 1975; Stewart, 1975). However, protrusion is not evident at flowering in some fiber development mutant lines. Lee et al. (2006) observed fiber initiation retardation in the dominant naked seed mutant line N1. Ruan et al. (2005) found the down-regulation of sucrose synthase and closure of plasmodesmata as related to fiber initiation retardation and slower rate of fiber elongation using lintless mutant stock (*Gossypium hirsutum*) acc. SL1-7-1 as material, with wild type cotton (*G. hirsutum*) var. Coker 315 as control.

Past research has focused on fiber initiation, and a series of genes related to this stage were cloned and characterized. *GhMYB1–GhMYB6* (Loguercio et al., 1999) were involved in fiber cell differentiation, while fiber initiation and anthocyanin biosynthesis were attributable to *GhTTG1–GhTTG4* (Humphries et al., 2005). *MYB25* also contributed to fiber initiation (Wu et al., 2007), whereas *expansin* (*EXP1*) played a role in fiber elongation (Harmer et al., 2002). Yang et al. (2006) compared ESTs of fiber cells with those from other tissues and found that genes encoding MYB and WRKY transcription factors were enriched in the early stages of fiber and ovule development. Fiber cell elongation actually composed of two distinct stages of expansion, and the early stage of fiber expansion, often called initiation, began with the formation of fiber initials (Wilkins et al., 2005). It was reported that reactive oxygen species

Abbreviations: DPA, day post-anthesis; EST, expressed sequence tag; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; SEM, scanning electron microscope; TM-1, texas marker-1; XinFLM, Xinxiangxiaoji Linted-Fuzzless Mutant

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(ROS) such as superoxide radical, hydrogen peroxide (H_2O_2), and hydroxyl radical were found to participate in plant cell expansion (Schopfer, 2001; Rodriguez et al., 2002; Liszkay et al., 2004). The control of intracellular ROS level is thought to be essential to maintain cellular redox homeostasis (Mittler et al., 2004), and an increase in ROS production was related to the initiation of root hairs (Frahry and Schopfer, 1998). Further, wild-type cotton ovules treated with exogenous H_2O_2 or ethylene *in vitro* could induce extended fiber cell elongation (Li et al., 2007). So, the genes participating in fiber initiation may be correlated with the ROS. Moreover, plant growth hormones, including IAA, GA3, and ABA, also affect cotton fiber development *in vitro* (Beasley, 1973; Beasley and Ting, 1974; Du and Pan, 2000; Sonal and Vrinda, 2002). Kim and Triplett (2001) observed that the fiber on the day of anthesis ovules could initiate in cultures supplemented with both IAA and GA3, but cytokinins, ABA and ethylene inhibited the fiber development, which indicated that phytohormone played important roles in fiber development. During the last decade, characterization of the cotton fiber transcriptomes (Arpat et al., 2004) has enriched our understanding on the molecular mechanism of fiber development, and some successes to genetically manipulate fiber growth have been attained (Wilkins and Jernstedt, 1999; Wilkins et al., 2001). Nevertheless, the molecular mechanisms of fiber initiation, and the relationship between genes and fiber initiation, and the effects of hormones on fiber initiation are still not clear.

Yield and quality of mature cotton fiber are closely correlated with the timing of fiber initiation and the number of initiated fibers (Ruan and Chourey, 1998). Therefore, elucidation of the mechanisms of fiber initiation is valuable to the cotton industry. Previously, we (Zhang et al., 2007) observed that fiber initiation was delayed in some mutants based on SEM. In this study, two mutants displaying delayed initiation, N1 and XinFLM, were further examined by SEM to compare developmental differences. Reverse transcription-polymerase chain reaction (RT-PCR) was also conducted using *in vitro* cultured XinFLM ovules to evaluate the expression of fiber-related genes and to explore the factors affecting fiber development. Further, a series of treatments on ovules with H_2O_2*in vivo* and BT medium supplemented with IAA and GA3 *in vitro* were applied to identify factors affecting fiber initiation retardation. This paper will report the results obtained from these experiments.

Materials and methods

Plant materials

Three Upland cotton (*Gossypium hirsutum* L.) fiber development mutants with lint but no fuzz, i.e., recessive naked seed (n2), dominant naked seed (N1), and Xinxiangxiaoji Linted-Fuzzless Mutant (XinFLM), were used in this study, with Upland cotton genetic standard line texas marker-1 (TM-1), with lint and fuzz as control. Their seed and fiber phenotypes were shown in Fig. 1. Of these, TM-1, N1, and n2 were from USDA-ARS (College Station, TX, USA), and XinFLM was bred by Cotton Research Institute, Nanjing Agricultural University (NAU). All materials were grown at Jiangpu Experiment Station, NAU. Treatment began, at 7:30–8:30 a.m. of August 4, 2008. Two milliliters of 30% H_2O_2 were *in vivo* injected into N1 and XinFLM ovules on the day before anthesis (−1 DPA) for 8, 16, 24, 48 h, with injection of 2 mL H_2O as mock-treatment, and *in vitro* culturing of ovules with acidic BT medium (pH 3–4) and BT medium supplemented with IAA (10 μM) and GA3 (10 μM) for 24 h. The treated ovules from at least ten immature ovaries for each treatment and three independent repeats for each genotype were sampled. All treated ovules were fixed in 3% glutaraldehyde (pH 7.2) at 4 °C for SEM analysis. XinFLM ovules were treated with

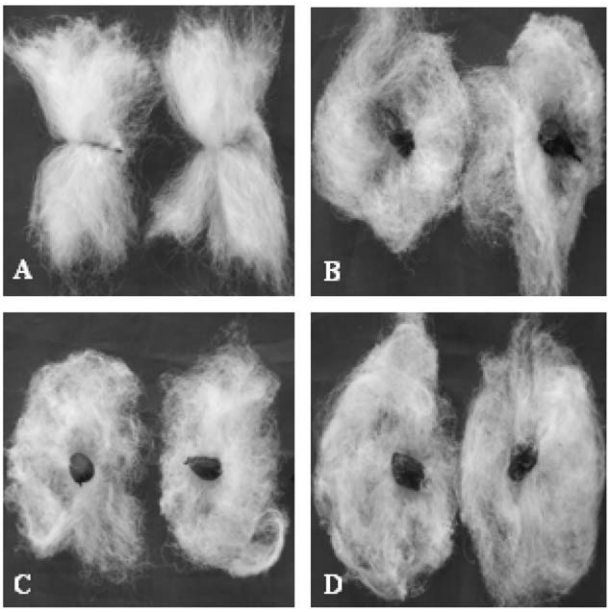

Fig. 1. The seed and fiber phenotypes in four Upland cotton accessions. A: TM-1, with lint and fuzz; B: n2, with lint and no fuzz; C: N1, with lint and no fuzz; D: XinFLM, with lint and no fuzz.

30% H_2O_2 for 24 h (equal to 0 DPA) and 48 h (equal to 1 DPA) and then immediately frozen in liquid nitrogen and stored at −70 °C for RT-PCR.

Sample preparation for SEM analysis

Ovule samples were first dehydrated serially for 30 min each in 30%, 50%, 70%, 80%, 90%, and 100% ethanol solutions. They were then placed in isoamyl acelate three times for 30 min each. Thereafter, samples were freeze-dried (Hitachi ES-2030, Tokyo, Japan) and sputter-coated with silver using an ion sputter (Hitachi E-1010/E-1020), then the specimens were observed using an SEM (Hitachi S-3000N). Each specimen was observed at an accelerating voltage of 15 kV, and images were stored as TIF files.

Total RNA extraction and RT-PCR analysis

Total RNA was extracted using the CTAB method (Jiang and Zhang, 2003). The first-strand cDNA was synthesized by avian myeloblastosis virus (AMV) transcriptase (Promoga, USA). One microgram of RNA was used to produce cDNA according to the manufacturer's instruction. A 10-fold dilution of the reaction product served as template for RT-PCR analysis, which was performed under the following conditions: 3 min at 94 °C; followed by 26–27 cycles of 45 s at 94 °C, 45 s at 55 °C, and 1 min at 72 °C. After the last cycle, the amplification was extended for 5 min at 72 °C. Three independent RT-PCR experiments were carried out, with constitutive expression of the elongation factor 1 alpha gene (*EF1α*) as a control. The polymerase chain reaction (PCR) product was separated on a 1.0% agarose gel. The genes used in this study included *MYB1*, *MYB5*, *MYB6*, *MYB25*, *MYB36*, *TTG1*, *TTG2*, *TTG3*, *TTG4*, *E6*, and *EXP1*. The primers used were listed in Table 1. The relative expression quantities of *MYB25*, *TTG1*, *TTG3*, and *EXP1* genes were further measured using qRT-PCR.

Real-time quantitative RT-PCR

The cotton *EF1α* was amplified as a reference to the target gene for gene expression. PCR products were detected by SYBR Green I