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Levels of Cytokinins in the Ovules of Cotton Mutants with Altered Fiber Development

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Abstract. Endogenous levels of cytokinin and abscisic acid (ABA) were determined in ovules of normal cotton (TM-1) and four fiber differentiation mutants (n2, Ligon lintless, H10, and Xu142) before and after flowering by enzyme-linked immunosorbent assays. The fluctuation patterns of ABA levels in ovules of normal cotton and mutants were similar. At the fiber elongation stage, ABA content was low, and from 1 day after flowering, the ABA content decreased steadily. On the other hand, the peaks of isopentenyladenine and isopentenyladenosine in ovules of TM-1 were observed 1 day before flowering. The level of cytokinins decreased after flowering in TM-1, whereas in the mutants it increased steadily. These results indicate that endogenous ABA is probably not the main inhibitor for fiber elongation and that endogenous cytokinins likely play a dual role in fiber development. Before flowering, cytokinins function as one of the stimuli for the initiation of fibers, but after flowering, cytokinins inhibit fiber growth.

Key Words. Cotton fiber—Cytokinin—Abscisic acid—Cotton ovule—Mutant

The cotton fiber is a single cell derived from the epidermis of cotton ovules. Fiber length is one of the most important fiber quality parameters. Since both fertilized and unfertilized cotton ovules can be cultured in vitro (Beasley 1971, 1973, Beasley and Ting 1973, 1974), a number of studies have focused on the effects of exogenous plant growth regulators on fiber development in tissue culture. It is generally accepted that exogenously applied IAA and GA enhance the differentiation of fibers and promote their elongation, whereas ABA and cytokinin inhibit fiber growth (Beasley and Ting 1973, 1974, Chen et al. 1988, Shen et al. 1978, Wang et al. 1985, Zhang 1982, Zheng and Xu 1982). However, little progress has been made in the characterization and the quantification of endogenous hormones in cotton ovules. ELISA may be used in such analysis (Weiler 1986). Previously, we have determined the levels of endogenous plant hormones in ovules of normal cotton (TM-1) and a fiber differentiation mutant (Xin) and found that a high level of IAA before flowering may initiate the fiber production and that 13-hydroxy-GAs may promote fiber elongation (Chen et al. 1996). Interestingly, the level of cytokinins in the ovules of mutant cotton was found to be extremely high. This indicates that endogenous cytokinins have an inhibitory effect on fiber development. Fiber differentiation mutants, which differ from each other in the appearance of lints and fuzz, offer a very useful experimental model for probing the roles of plant hormones in fiber development. Here we report the levels of endogenous cytokinins in four fiber differentiation mutants before and after flowering, and we discuss their relation to fiber development.

Materials and Methods

Plant Materials

Cotton (*Gossypium hirsutum* L.) ovules in normal (TM-1, with normal lints and normal fuzz, used as control) and mutant plants, n2 (with normal lints and fuzzless), Ligon lintless (Li, with very short lints and normal fuzz), H10 (with sparse lints and fuzzless), and Xu142 (lintless and fuzzless), were collected 3 days and 1 day before flowering and 0, 1, 3, 5, and 8 days after flowering, respectively. Fresh samples were

Abbreviations: IAA, indole-3-acetic acid; GA, gibberellin; iPA, isopentenyladenosine; ZR, zeatin riboside; DHZR, dihydrozeatin riboside; ABA, abscisic acid; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; FW, fresh weight; S.E., standard error.

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weighed, placed in liquid nitrogen, and kept at −20°C until extraction for hormone analysis

Extraction and Measurement of Cytokinins and ABA by ELISAs

Extraction and purification of cytokinins and ABA prior to immunoassay have been described previously (Chen et al. 1996, 1997a, 1997b, Wang et al. 1994, Zhang et al. 1995): extraction of homogenized samples in cold 80% (v/v) aqueous methanol at a rate of 5 mL/g FW overnight at 4°C with butylated hydroxytoluene (10 mg/liter) to prevent oxidation. The supernatant was collected after centrifugation at 10,000 ×*g* (4°C) for 20 min. Then, the crude extract was passed through a C_{18} Sep-Pak catridge (Waters, Milford, MA). The efflux was collected, and 600 μL was removed and dried in N_2. The residue was dissolved in 600 μL of PBS (0.01 M, pH 7.4) for determining the levels of iPAs, ZRs and DHZRs, respectively. Another 200-μL aliquot of the filtrate was taken and dried in N_2, and the residue was dissolved in 200 μL of PBS (0.01 M, pH 9.2) and adjusted to pH 8.5 before partitioning three times with equal volumes of ethyl acetate. The remaining aqueous phase was adjusted to pH 2.5 and extracted three times with equal volumes of ethyl acetate. The extracts (ethyl acetate phase) were pooled and dried in N_2; the residue was redissolved in 200 μL of 100% methanol for methylation with freshly synthesized ethereal diazomethane and taken up with 200 μL of PBS for ABA ELISA.

The indirect competitive ELISA measurement using polyclonal antibodies against iPA, ZR, and DHZR has been described by Chen et al. (1992), Chen and Zhou (1996), and Wang et al. (1994). Microtitration plates (Nunc) were coated with synthetic cytokinin-ovalbumin conjugates in $NaHCO_3$ buffer (50 mM, pH 9.6) and left overnight at 37°C. Ovalbumin solution (10 mg/mL) was added to each well for blocking the nonspecific binding. After incubation for 30 min at 37°C, authentic cytokinin or sample and antibodies were added and incubated for 45 min at 37°C. Then horseradish peroxidase-labeled goat antirabbit immunoglobulin was added to each well and incubated for 1 h at 37°C. Finally, the buffered enzyme substrate (orthophenylenediamino) was added, and the enzyme reaction was carried out in the dark at 37°C for 15 min, then terminated using 3 M H_2SO_4. The absorbance was recorded at 490 nm. Each of the cytokinin antibodies displayed significant cross-reactions with its corresponding free base, and the results were therefore expressed as the combined contents of iPA plus isopentenyladenine, ZR plus zeatin (Z), and DHZR plus dihydrozeatin (DHZ), respectively.

The procedure of direct competitive ELISA measurement based on monoclonal antibodies of high specificity for ABA methyl ester (showed no significant cross-reactions with other naturally occurring plant growth regulators) has been described by Zhou et al. (1996). Microtitration plates were precoated overnight at 4°C with rabbit antimouse immunoglobulin. Then the wells were coated with suitable amounts of monoclonal antibodies in PBS (0.01 M, pH 7.4) at 37°C for 70 min. Authentic hormone or sample was added 1 h before the addition of horseradish peroxidase-labeled hormone. After a 2-h incubation at 25°C, the substrate for the enzyme was added, and the absorbance at 490 nm was recorded as above.

The need to validate immunoassays for plant hormones has been well documented (Pengelly 1985). In this study the percentage recovery of each hormone was calculated by adding known amounts of authentic hormone to split extract. Percentage recoveries were all above 90%, and all samples extract dilution curves paralleled the standard curves, indicating the absence of nonspecific inhibitors in the extracts. Each hormone was determined three times on the same extract, and all samples were assayed in triplicate. The S.E. was calculated.

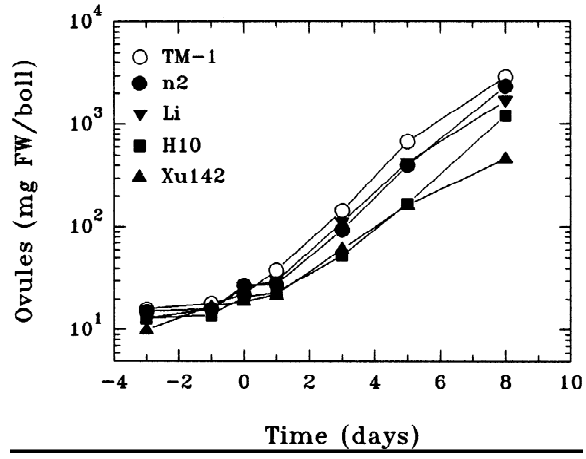

Fig. 1. Changes in FW of ovules/boll of normal (TM-1, with normal lints and normal fuzz, used as control) and mutant cottons, n2 (with normal lints and fuzzless), Ligon lintless (Li, with very short lints and normal fuzz), H10 (with sparse lints and fuzzless), and Xu142 (lintless and fuzzless), during flowering. Shown are the means of 20 bolls. Days before and after flowering are shown as negative and positive numbers, respectively.

Results

Changes in Fresh Weight of Cotton Ovules during Flowering

From −3 days (3 days before flowering) to +8 days (8 days after flowering), a significant increase in the FW of ovules was observed after flowering (Fig. 1). Before flowering, no major difference in the FW of ovules was observed between normal and mutants. From 0 to +1 day, the FW of the ovules increased by 70% in TM-1 but only less than 15% in mutants. From +1 day to +3 days, the FW of the ovules increased 2.8-fold, 2.3-fold, 2.7-fold, 1.3-fold, and 1.7-fold in TM-1, n2, Li, H10, and Xu142, respectively. From +3 days to +5 days, it increased 3.7-fold, 3.2-fold, 2.8-fold, 2.1-fold, and 1.6-fold, respectively. Eight days after flowering, the FW of the ovules of TM-1 was more than twice that of H10 and more than six times that of Xu142. This increase of FW of ovules was positively correlated with the process of fiber development (except in Xu142), and the FW of ovules was positively correlated with the degree of fiber development around ovules.

Changes of Endogenous ABA Level in Cotton Ovules during Flowering

Fluctuation patterns of ABA levels in ovules of normal and mutant cottons are shown in Fig. 2. The peak of ABA in the ovules of TM-1 was observed 1 day after flowering. A similar peak was observed in the ovules of

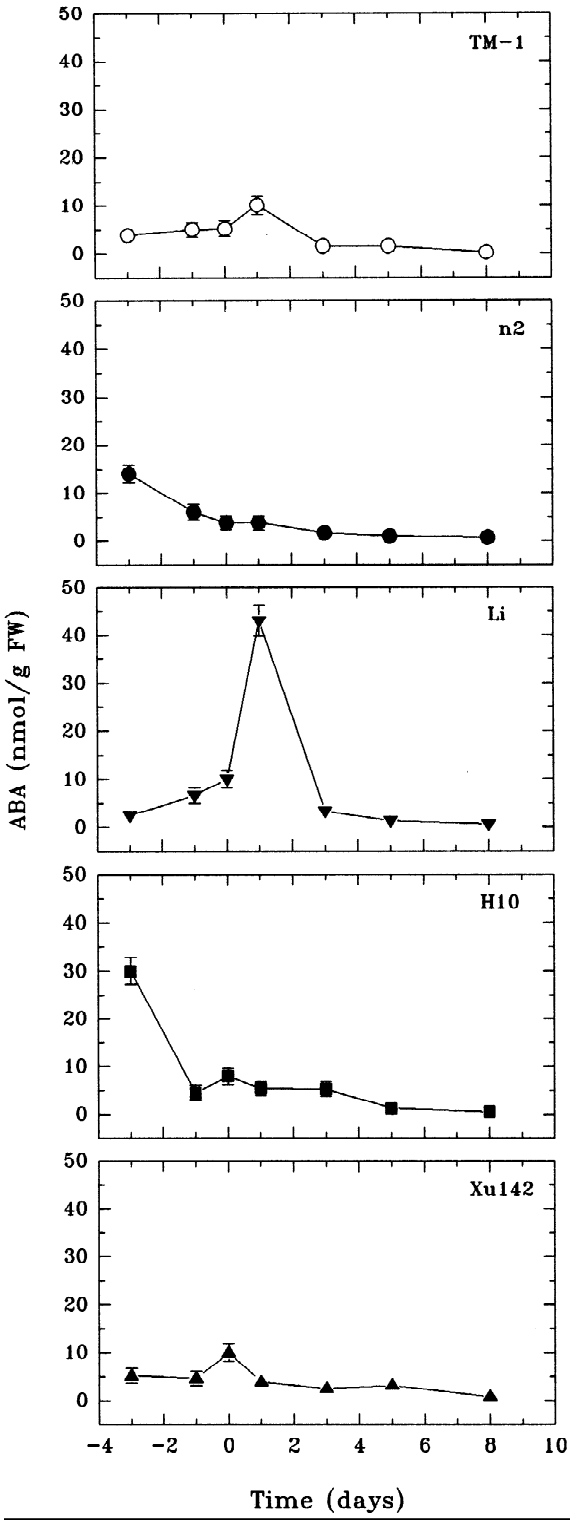

Fig. 2. Changes in endogenous ABA levels in ovules of normal (TM-1) and mutant cottons, n2, Li, H10, and Xu142, during flowering. Shown are the averages ± S.E. of three replicates. Days before and after flowering are shown as negative and positive numbers, respectively.

Li, but it was more than four times higher than that in TM-1. The highest ABA content was determined 3 days before flowering both in n2 and in H10, and the amount of ABA in H10 was twice that in n2. The peak of ABA content in Xu142 was observed when flowering. However, from +1 day, the ABA contents both in TM-1 and in the mutants decreased steadily, and at the fiber elongation stage (3 days after flowering), ABA content was very low.

Changes of Endogenous Cytokinin Levels in Cotton Ovules during Flowering

Cytokinins were very abundant in cotton ovules, and extremely high levels of cytokinin in mutants were determined after flowering (Fig. 3)

The peak of iPA content was observed at −1 day in TM-1, after which it decreased by almost 95% when flowering and decreased by 90% 24 h after flowering. Eight days after flowering, it decreased to 0.2 nmol/g FW. The peak of iPA content in Li was observed at 0 day, and an increase in iPA content was observed from +1 to +5 days. The iPA content increased steadily in n2, H10, and Xu142 from 0 to +5 days, and a low level of iPA was determined at +8 days (Fig. 3). It was interesting to note that 3 and 5 days after flowering, endogenous levels of iPA were negatively correlated with the FW of ovules (Fig. 3).

One day before flowering, ZRs in ovules of TM-1 were found to have a higher level compared with those of mutants, and no obvious change in the levels of ZR in ovules of TM-1 was observed from −3 to +5 days with the range of ZR content being 1.8–3.7 nmol/g FW. Similar fluctuation was observed in n2. However, in Li, a peak of ZR content was observed when flowering. The content of ZR in H10 and Xu142 increased steadily after flowering (Fig. 3).

The peak of DHZR content was observed at +1 day in TM-1, after which it decreased by 85% within 48 h and was found to be only 1.0 nmol/g FW at +8 days (Fig. 3). However, in n2, Li, and H10 the DHZR levels increased consistently from +3 to +8 days. In Xu142, the DHZR levels increased from −3 days. It was also noted that from +3 to +8 days endogenous levels of DHZR were negatively correlated with the FW of ovules and the degree of fiber development around ovules (Fig. 3).

Discussion

In cotton, the initiation of fibers starts before flowering and fertilization, and fibers elongate during anthesis (Beasley 1973, Stewart 1975). Since in the absence of IAA and/or GA, fiber elongation does not occur, IAA and GA have been thought to be the two main promoters

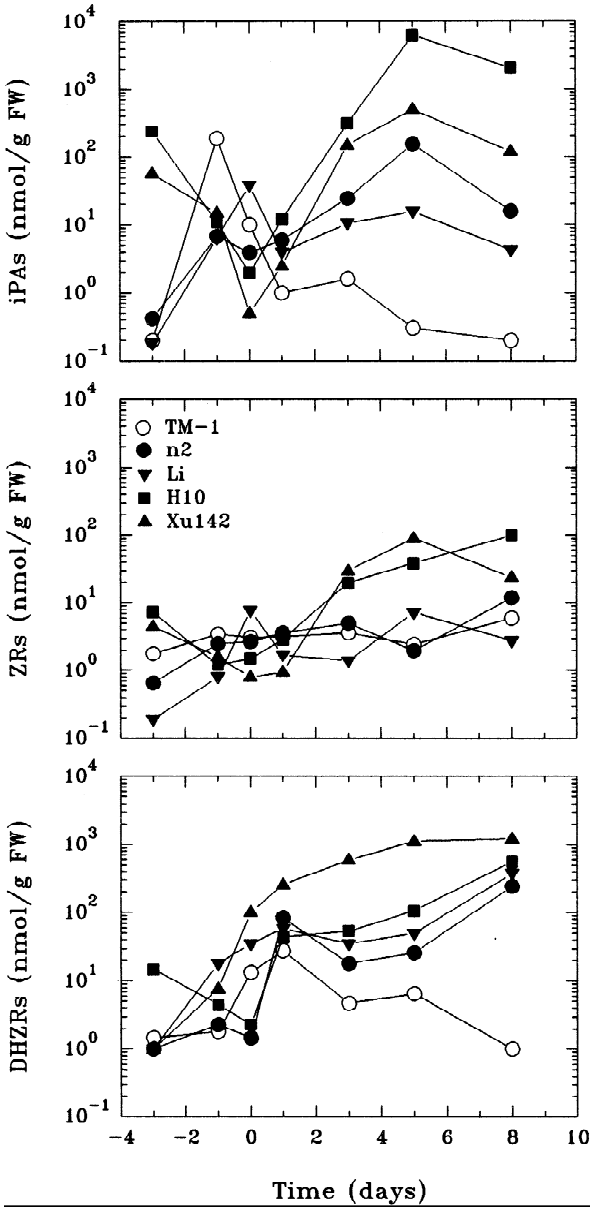

Fig. 3. Changes in endogenous cytokinin levels in ovules of normal (TM-1) and mutant cottons, n2, Li, H10, and Xu142, during flowering. Shown are the averages of three replicates. Days before and after flowering are shown as negative and positive numbers, respectively.

of fiber development (Beasley and Ting 1973, 1974, Chen et al. 1988, Shen et al. 1978, Zhang 1982). ABA can reduce the capacity of ovule to produce fibers in the presence of IAA or GAs (Beasley and Ting 1974), so ABA may be one of the inhibitors of fibers development. In the present study, we report that 3 days after flowering (fiber elongation stage) the ABA content in ovules of mutants (Li, H10, and Xu142) with abnormal lints (or lintless) is about twice that of normal cotton, TM-1, and mutant (n2) with normal lints (Fig. 2). These data confirm the hypothesis mentioned above that a high level of ABA may inhibit fiber production. However, from +1 day, the ABA content both in TM-1 and in the mutants decreases steadily; and at the fiber elongation stage, the ABA content is very low (Fig. 2). In addition, inhibitory effect of exogenous ABA is similar in both fertilized and unfertilized ovules grown in vitro (Beasley and Ting 1973, 1974). In this case, mutation in lint or fuzz cannot be explained only by the level of ABA. This result implies that endogenous ABA is not likely to be the major inhibitor of fiber development and indicates the presence of other inhibitors.

It has been reported that fiber development stimulated by IAA or GA is reduced by a high concentration of cytokinin, whereas a low concentration of cytokinin partially reverses the inhibitory effect of ABA (Beasley and Ting 1974). In addition, major differences in the effect of cytokinin on fertilized and unfertilized ovules have been noted (Beasley and Ting 1973, 1974). In fertilized ovules, the level of endogenous cytokinin is likely optimal (at least for fiber development) since exogenously applied cytokinin has only an inhibitory effect on fiber production. In unfertilized ovules, cytokinin provides for some growth of the immature seed but does not provide for any fiber development at any concentration used. These results imply that the effect of cytokinin on fiber development may be mostly dependent on its level and the stage of fiber development. In the present study, extremely high levels of cytokinin in the ovules of mutant are determined after flowering (Fig. 3), but the FW of ovules in mutants is significantly lower than that of TM-1 (Fig. 1). If we take into consideration the fact that the increase in weight of ovules is positively correlated with both the process and the degree of fiber development around the ovules, it can be concluded that excessive endogenous cytokinins reduce the capacity of the ovule to produce fibers or to increase in size. Cytokinins may, therefore, be the main inhibitors to fiber production in the ovules of mutants.

We also find that 1 day before flowering the levels of iPA and ZR in ovules of TM-1 are significantly higher than those of mutants, and the peak of iPA levels is observed (Fig. 3). Since the initiation of fibers starts before flowering (Beasley 1973, Stewart 1975), it is possible that a relatively high concentration of cytokinin may have provided for an increase number of fiber initials.

Take together, we suggest that before flowering, cytokinins may function as one of the stimuli for the initiation of fiber, but the subsequent elongation of the fibers requires low levels of cytokinin, and high levels of cytokinin inhibit fiber growth.

We do not know yet exactly why cotton fiber differentiation mutants can accumulate cytokinins in their ovules and whether the enhanced cytokinin levels result from reduced cytokinin oxidase-mediated degradation as

a result of reduced auxin levels (Hare and van Staden 1994). A more careful analysis of endogenous hormones is now underway.

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