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Early Development of Duplication-Deficiency Ovules in Upland Cotton[1]

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ABSTRACT

Viable duplication-deficiency (dp-df) products are recovered from heterozygous translocations in Upland Cotton (*Gossypium hirsutum* L.) with an overall frequency high enough to warrant their use in cytogenetic experiments. The frequencies of dp-df's from most translocations, however, are considerably lower than predicted from meiotic configurations. Heterozygotes (Tt) of two translocation lines were studied to determine the period of development during which dp-df's were lost. In Tt2;14 2B-1, which normally yields a high frequency of dp-df's, the frequency of ovule failure was similar to that in TM-1 controls. Failure of ovules began at 5 days after anthesis in Tt10;11 2785. A high percentage of ovules from Tt10;11 at 5 days postanthesis were found with intact synergids and polar nuclei, indicating that failure may occur during fertilization. Endosperm failure may also be responsible for some dp-df loss, because, in 4% of ovules from Tt10;11, nucellar cells filled the interior of the ovule and obliterated the space normally occupied by embryo and endosperm. Attempts were made to enhance the recovery of dp-df's by culture of 2-day-postanthesis ovules. In both lines, the frequency of dp-df's was significantly higher ($P = 0.001$) in the cultured progenies than in controls. In ovulo culture also decreased by 50% the time required to obtain seedlings.

Additional index words: **Cotton, Duplication-deficiencies, Ovule culture, Development, Cytogenetics, Embryo rescue.**

UPLAND COTTON (*Gossypium hirsutum* L.) is an allotetraploid ($2n = 52$) (Beasley, 1942). It has become meiotically diploidized so that all chromosomes pair as bivalents at metaphase I. Fifty-eight homozygous translocation lines (TT) have been developed that involve reciprocal translocations between two pairs of chromosomes (Brown, 1980). Twenty-five of the 26 chromosomes are involved in one or more of the translocations. Plants heterozygous for a translocation (Tt) produce both balanced gametes and unbalanced duplication-deficiency (dp-df) gametes at meiosis, depending upon the type of metaphase I orientation and subsequent disjunction of the quadrivalent (IV).

Most types of dp-df's arising from adjacent-1 segregation are ovule-viable in cotton (Menzel et al., 1986), and they can be used similarly to monosomes or telosomes in cytogenetic experiments (Endrizzi et al., 1985). Most translocation lines, however, yield fewer dp-df plants than would be expected from metaphase I orientations of the chromosomes in the translocation IV (Menzel and Brown, 1952, 1978; Brown et al., 1981; Menzel and Richmond, 1983; Menzel et al., 1986). Meiotic orientations have not been determined for embryo sac mother cells (megasporocytes) in cotton, but experience has given no reason to suspect that they differ substantially from those of PMC's (microsporocytes) (Menzel, unpublished). From some translocations, dp-df plants are rarely obtained, despite a presumably high primary frequency of dp-df megaspores. In this study, the developmental stage at which dp-df's were lost was investigated in two translocation lines (Contolini, 1985).

The technique of in vitro ovule culture is successful in rescuing many interspecific cotton hybrid embryos (Hu and Li, 1982; Sheng-Zhang, 1983; Gill and Bajaj, 1984; Refaat et al., 1984). Anticipating the significance of rescuing plants with chromosomal deficiencies and duplications, the authors attempted to enhance the frequency with which dp-df's were recovered by in vitro culture of young ovules from plants expected to yield dp-df progeny.

MATERIALS AND METHODS

Two translocation lines were used, T2R;14R 2B-1 and T10R;11R 2785. T2;14 was chosen because in earlier experiments heterozygotes yielded close to the predicted (i.e., postmeiotic) frequency (49%) of dp-df progeny (Menzel and Brown, 1952). In contrast, heterozygotes of T10;11 yielded only 6% dp-df's (Menzel et al., 1986), though the predicted frequency is about 55% (Brown et al., 1981). Plants homozygous (TT) for these two translocations were crossed with the standard line TM-1 (tt) (Kohel et al., 1970) to yield heterozygous plants (Tt). When backcrossed as ovule parents to TM-1, the heterozygotes produced progeny that contained tt, Tt, and dp-df plants. Primarily, the BC1 generation was used for this study.

Ovule Sections

Ovaries were removed from field-grown plants throughout the blooming season and fixed for 3 days in a 3:1 mixture of ethanol/acetic acid. After removal from the fixative, the ovary walls were slit to expose the ovules, which remained attached to the placenta. The ovaries were then passed through an alcohol dehydration series and embedded in paraffin wax. Sections were 10 μm thick and affixed to slides coated with Haupt's adhesive. Mounted sections were stained with 1% safranin in absolute ethanol or with 1% aqueous safranin (for lignin, nucleoli, and chromosomes) and counterstained with 0.5% fast-green in a mixture of 50% clove oil/50% ethanol or with 1% aniline blue in absolute ethanol (for cytoplasm and cellulose). Stained sections were examined and some were photographed with a Zeiss Photomicroscope II with an apochromatic 10X objective and 1.25 optovar setting. Kodak Panatomic X or Plus X film was used at an ASA setting of 32.

Ovule Culture

Ovaries at 2 to 6 days postanthesis were surface-sterilized by immersion in undiluted Physan 20 (Consan Pacific Inc., Whittier, CA), dipped in 95% ethanol, and flamed. To establish seedlings, we cultured ovules according to the method of Stewart and Hsu (1977). Young plants were transplanted to the experimental cotton garden at the Mission Road Research Facility, Florida State Univ. To generate controls, we germinated seeds in peat pellets and transferred them to the garden.

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Dp-df Analysis

To determine the frequency of dp-df plants from the cultured progeny, we peeled floral buds from mature plants to expose the staminal column and fixed them in a fresh mixture of 3:1, 95% ethanol/glacial acetic acid. Pollen mother cells (PMC) were stained with iron-acetocarmine and squashed on slides for light microscopic analysis. Cytotypes were determined from metaphase I configurations according to previously published criteria (Menzel, 1955; Menzel et al., 1986). Morphological traits of plants were also helpful in detecting dp-df's from Tt10;11 (Menzel et al., 1986).

RESULTS AND DISCUSSION

On the day of anthesis, ovules dissected from ovaries of TM-1, TT10;11, Tt10;11, TT2;14, and Tt2;14 plants appeared smooth, white, and round, with a diameter of about 0.5 mm. At 5 days postanthesis, some ovules of the Tt10;11 plants had stopped developing and had begun to degenerate. The degenerating ovules appeared shriveled and discolored, whereas others, as well as those of Tt2;14, and TM-1, reached a diameter of 1 mm. No significant increase in the number of degenerating ovules occurred thereafter (Contolini, 1985). Percentages of normally developed ovules per ovary were calculated for all lines from 5 to 45 days postanthesis (Table 1). The *t* tests indicated that the Tt10;11 plants had significantly lower percentages of normally developed ovules than all other materials ($P < 0.01$). The total number of ovules per locule was also calculated for each line to determine whether the T10;11 line was inherently producing fewer ovules (Table 1). Though *t* tests indicated that the homozygous TT10;11 plants were producing fewer ovules than the others ($P < 0.01$), the heterozygous Tt10;11 plants had ovule numbers similar to those of TT2;14, Tt2;14, and TM-1 plants.

Because 5 days postanthesis was the earliest time at which ovule failure was detected, sectioned material from ovules at that stage was compared with that from the day of anthesis. In sectioned ovules fixed on the day of anthesis, the embryo sac appeared as a vacuolate structure located in the center of the nucellus. The egg cell was flanked by two synergids, and the two polar nuclei were sometimes visible towards the center of the gametophyte. The antipodal cells were not usually visible, because they normally disappear 7 days prior to anthesis (Gore, 1932; Jensen, 1965). Ovules from Tt2;14, Tt10;11, and TM-1 plants all had this internal morphology.

At 5 days after anthesis, the internal morphology of the ovules fell into four categories (Table 2). The majority of ovules (Type I) contained free-nuclear endosperm and embryo (Fig. 1). The diameter of the embryos ranged from 10 to 30 μm. Presumably these ovules resulted from normal fertilization.

Type II ovules contained free-nuclear endosperm but lacked embryos. The structure of the endosperm appeared similar in ovules with and without embryos. In both cases it lined the embryo sac wall, was noncellular, and contained multiple nucleoli (Fig. 2). (Jensen et al. [1977] reported that unfertilized cotton ovules contained diploid nuclei that became cellular on the 3rd day after anthesis, but only about six nuclei formed.) We surmise that these ovules lacked embryos for reasons other than blocked fertilization. Two possible explanations include (i) loss of embryos during sectioning and (ii) partial fertilization, in which a sperm nucleus fused with the polar nuclei but not with the egg.

Eleven ovules of Tt10;11, two of Tt2;14, and two of TM-1 contained a vacuolate gametophyte without endosperm (Type III). In these cases, the cavity of the embryo sac appeared smaller than in ovules containing endosperm. Usually, the two synergids could be seen at the micropylar end, while the two polar nuclei were sometimes seen near the center (Fig. 3). Remnants of the egg cell could sometimes be detected in nearby sections of the same ovule. They resembled unfertilized TM-1 ovules (Fig. 4 and 5). It is possible that fusion of the egg and sperm is inhibited in certain dp-df embryo sacs.

In two ovules of Tt10;11, endosperm was absent and the area that should have been occupied by the embryo sac was filled with nucellar cells (Type IV) (Fig. 6). This condition was not observed in Tt2;14 or TM-1. They may have been the result of abnormal endosperm development, gametophyte abortion, or something resembling the "somatoplastic sterility" described in *Nicotiana* (Cooper and Brink, 1940) and *Solanum* (Lee and Cooper, 1958).

In sectioned ovules of Tt10;11 at 5 days postanthesis, the percentage of ovules that appeared either unfertilized or completely filled with nucellar tissue was similar to the 36% of degenerated ovules in ovaries dissected at the same age. Incomplete fertilization alone cannot account for this degeneration because it was not observed in unfertilized TM-1 ovules. Some Tt10;11 dp-df gametophytes seem to express developmental blocks that reduce fertilization and hasten degeneration.

Table 1. Percentages of normally developed ovules per ovary and total numbers of ovules per locule in cotton at 5 to 45 days post anthesis.

Material	Percent normal ovules/ovary $\bar{x}$	SD	*n*	Total ovules/locule $\bar{x}$	SD	*n*
TM-1 (tt) Selfed	95.68	± 5.65	19	8.66	± 0.37	19
TT10;11 Selfed	92.47	± 10.16	17	7.72	± 0.73	17
Tt10;11 × TM-1 (tt)	63.71	± 12.62	21	8.17	± 0.61	19
TT2;14 Selfed	88.42	± 12.94	21	8.65	± 0.76	11
Tt2;14 × TM-1 (tt)	85.90	± 22.52	11	8.90	± 0.64	21

Table 2. Types of cotton ovules sectioned at 5 days post anthesis according to internal anatomy.

Ovule types	Tt10;11 × TM-1 (tt) no.	%	Tt2;14 × TM-1 (tt) no.	%	Selfed TM-1 (tt) no.	%
Total ovules	45	--	44	--	23	--
Embryo and endosperm	20	44.4	30	68.2	18	78.3
Endosperm only	12	26.7	12	27.3	3	13.0
Egg apparatus and polar nuclei	11	24.4	2	4.5	2	8.7
Solid nucellus	2	4.5	0	0	0	0

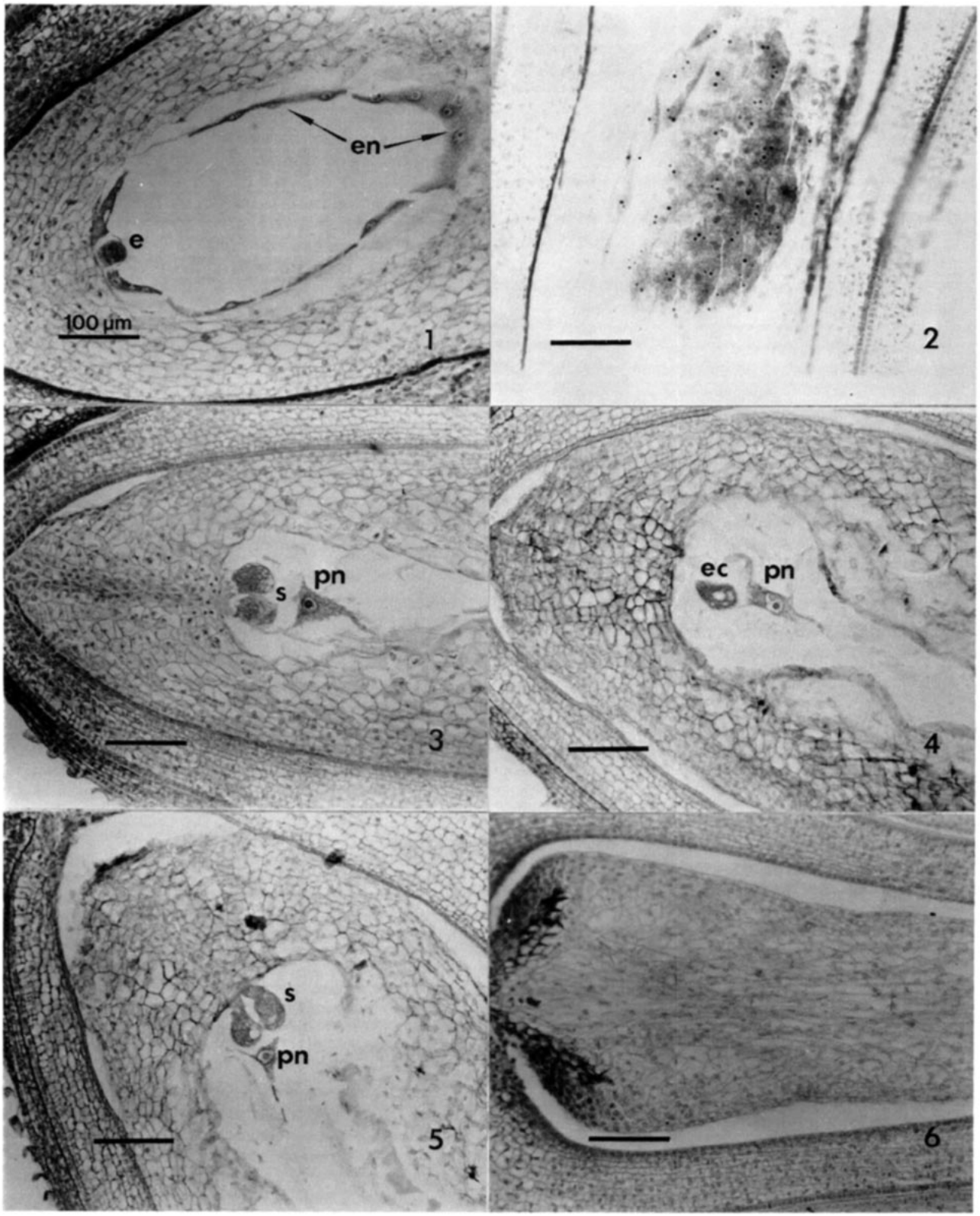

Fig. 1–6. Paraffin sections (10 µm) from cotton ovules fixed at 5 days postanthesis. *Fig. 1.* Ovule from a pollinated Tt10;11 flower showing embryo (e) and endosperm (en). *Fig. 2.* Layer of endosperm along inner wall of embryo sac in an ovule from a Tt2;14 plant. Multiple nucleoli are evident. *Fig. 3.* Ovule from a pollinated Tt10;11 flower. Polar nucleus (pn) and two synergids (s) are visible. The ovule appears to be unfertilized. *Fig. 4 and 5.* Unpollinated TM-1 ovules. Figure 4 shows the egg cell (ec) and polar nuclei (pn). Figure 5 shows two synergids (s) and polar nucleus (pn) (compare with Fig. 3). *Fig. 6.* Section through the midplane of an ovule from a pollinated Tt10;11 flower. Space normally occupied by embryo sac is filled with nucellar cells.

Table 3. Survival rates of cotton ovules cultured in vitro.

	No. ovules explanted	Ovules germinated		Plants transplanted to garden	
		no.	%	no.	% of germination
Tt10;11	382	112	29	16	14
Tt2;14	782	301	38	21	7

Table 4. Frequencies of cytotypes among progenies obtained from fertilization of translocation heterozygotes (Tt) with pollen from normal (tt) cotton. Ovules were grown in vivo (control) or in vitro (cultured).

Source	Total plants	tt		Tt		Dp-df	
		no.	%	no.	%	no.	%
Tt2;14 Control	57	22	38.6	22	38.6	13	22.8
Tt2;14 Cultured	21	10	47.6	1	4.8	10	47.6
Tt10;11 Control	43	19	44.2	24	55.8	0	0
Tt10;11 Cultured	16	5	31.2	9	56.2	2	12.5

Ovule Culture

Though 1164 ovules were cultured, only 37 seedlings survived to be planted in the field (Table 3). The culturing method needs some modification to improve the yield of mature plants. A major problem was that callus often proliferated from the micropylar end of the ovule, interfering with growth, and eventually covering the entire ovule. When such ovules were dissected, the embryo sacs appeared to have been crushed by the abnormally developed integuments, and embryo growth appeared severely retarded. Variations in temperature, N supply, and hormones may eliminate callus and thus increase the yield of healthy embryos. Stewart and Hsu (1978) found that altering the phytohormone levels in the growth medium, especially that of auxin, improved the yield of interspecific hybrid embryos between New World and Old World cotton plants. Adjustment of phytohormone and reduced NO_3^- levels may also aid in increasing the yield of dp-df embryos.

Another major period of loss occurred during the transition from incubator to greenhouse. At this time, the seedlings were subjected to a drastic change in relative humidity that resulted in high mortality despite precautionary measures.

Though the yield of mature cotton plants from cultured ovules was low in this study, it included dp-df, tt, and Tt plants (Table 4). Chi-square tests indicated that the percentage of dp-df's for the cultured progeny was significantly higher ($P < 0.001$) than that for the controls in both lines. However, only in the cultured Tt2;14 progeny did the observed frequency of dp-df's equal the frequency predicted by metaphase I configurations (Brown et al., 1981). The data suggest that culturing ovules in vitro increases the probability of recovering dp-df's.

In ovulo culture decreased the amount of time required to obtain seedlings. Normally, a cotton boll requires 50 to 60 days to mature, and the seeds must then be after-ripened for about 42 days for good germination. When the in vitro method was used, the average elapsed time between fertilization and germination was 38 days.

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