Dissimilar Behaviour of the Two Kinds of Duplication-Deficiency Female Gametophytes in the T3–6 Interchange Heterozygote of Pearl Millet (*Pennisetum glaucum* (L.) R. Br.)

K. Uma Devi*, T. V. Rajya Lakshmi, K. Aruna Lakshmi,
P. S. R. L. Narasinga Rao and K. Adisheshaiah

Department of Botany, Andhra University, Visakhapatnam–530 003, India

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Summary In structural heterozygotes for the T3–6 chromosomal interchange in pearl millet, the percent pollen fertility correlated with percent seed set. The duplicate-deficient (dp/df) microspores degenerated at binucleate stage. The effect of the dp/df condition on the structure and function of the female gametophyte was investigated in this interchange heterozygote. Inflorescences from the interchange heterozygote plants were fixed at five developmental stages:—at pre pollination (*i.e.* stage 1) and post pollination (*i.e.* stages 2 to 5) at 24 hr intervals up to 96 hr after pollination (hap). The embryo sac (female gametophyte) was dissected from the ovule and stained with 2% acetocarmine. In the post pollination samples the rate of early seed development was estimated by counting the number of cells in the embryo and endosperm. A normal looking embryo sac was found in all the ovules at pre pollination stage. Post pollination, two kinds of abnormalities were found in ~50% of the ovules: some had unfertilized embryo sacs; some showed a lag in development of the embryo and endosperm. These abnormalities could be due to the dp/df condition of the embryo sacs—the two kinds representing the two types of dp/df products formed in the interchange heterozygote. The difference in tolerance of the dp/df condition on the male and female sides is attributed to a) the (programmed) difference in the control of gametophyte development in the two sexes, and b) the existence of competition among the (three) microspore categories (normal, dp/df of one kind and dp/df of another kind) as opposed to the non-competitive aspect of megaspore development (which ever is the category).

Key words Pearl millet (*Pennisetum glaucum*), Duplication-deficiency spores, Female gametophyte, Embryo sac structure, Post pollination development, T3–6 interchange.

In structural hybrids heterozygous for a chromosomal interchange (reciprocal translocation), nearly half of the meiotic products are genetically imbalanced. The meiocytes in which the interchange multivalent shows an adjacent or indifferent orientation at metaphase produce spores which are duplicate for certain regions of a chromosome and deficient for some regions of another chromosome. These are described as duplication-deficiency (dp/df) spores. Two types of dp/df spores are produced from such meiocytes. The chromosomal region which is in duplicate in one kind of dp/df spores is the one for which the other kind is deficient and *vice-versa* (Fig. 1). The dp/df microspores fail to develop into normal male gametophytes (pollen) though they are physically present in a genetically balanced sporophyte. In such microspores, the development of the gametophyte is either not initiated or it is arrested midway leading to the formation of structurally abnormal pollen or, the gametophytes that develop from these microspores look normal but fail to compete with normal ones. The stage at which the development of the male gametophyte is affected depends on the chro-

* Corresponding author: Tel. No. 091 891 574871 Extn. 342. Fax No. 091 891 555547.

Fig. 1. Chromosome complement (with respect to the chromosomes that were involved in the interchange) of the normal and interchange heterozygote individuals and the *dp/df* meiotic spores.

mosomal region involved. Transmission of deleterious genetic condition to the next generation *via* the pollen is thus incidentally blocked. This beneficial effect of pollen selection is however diluted due to the absence of such a rigid selection on the female side. Deleterious genotypes and chromosomal aberrations that are occasionally transmitted to the offspring are most often carried through the female gamete (Khush 1973, Sybenga 1992). The extent to which *dp/df* condition affects female gametophyte development or function in comparison to the male gametophyte has not been analysed in almost all cases of the chromosomal interchanges studied in plants. There is a recent report (Ray *et al.* 1997) of the study of the female gametophyte in an interchange heterozygote of *Arabidopsis thaliana*. We have developed a technique of easy dissection of the embryo sac in pearl millet. We investigated the structure and functional ability of the female gametophytes in a structural heterozygote of the well characterized T3–6 interchange (Koduru and Krishna Rao 1984, Uma Devi *et al.* 1996) of pearl millet.

Approximately 50% of the microspores in the T3–6 interchange heterozygote of pearl millet degenerate after one pollen mitosis *i.e.* at the binucleate stage (Fig. 2a) and appear as ghosts in squashes made from mature anthers. These are assumed to be *dp/df* (Adiseshiah 1995). The percent pollen fertility correlated with the percent seed set in this interchange heterozygote in many genetic backgrounds (Koduru 1979). This indicates that *dp/df* products are formed in the same frequency on the male and female sides in the T3–6 interchange heterozygote. At what stage the female gametophyte development/function is affected by *dp/df* condition in the megaspores in this interchange heterozygote was investigated.

Material and methods

To get a population of plants heterozygous for the T3–6 interchange, a cross was made between the semidwarf T3–6 interchange homozygote line IP12781 and a genic male sterile segregant of a normal disomic inbred line Vg272. Eighty plants from the crossed seed were raised. Five of these plants picked at random possessed interchange multiples in pollen mother cells at diakinesis; the sample of 80 plants is thus taken to be a group of interchange heterozygotes.

To study the structure and functional competence of the female gametophytes in the interchange heterozygote, ears were fixed at five developmental stages. Bagged ears with styles fully emerged and ready for pollination were fixed to represent pre-pollination stage. For post pollination developmental stages, ears were artificially pollinated with fertile pollen from the disomic inbred Vg272. They were fixed post pollination at 24 hr intervals up to 96 hap to represent four developmental stages at 24, 48, 72 and 96 hap.

Two ears for each developmental stage were analysed. About 125 ovaries were dissected out from the florets of each ear. The ovary was floated in a drop of water on a slide, the entire embryo sac along with the antipodal cluster was dissected out with fine needles under a stereo dissection microscope with 50 $\times$ magnification. The embryo sac was stained with a drop of 2% acetocarmine and a coverslip applied for examination under a compound microscope.

In the post pollination samples, the rate of early seed development was estimated from ten ovules per sample. The criterion was direct count of the number of cells/nuclei in the embryo and endosperm. In pearl millet, cell (embryo) and nuclear (endosperm) divisions are synchronous up to 48 hap in the embryo and 72 hap in the endosperm. In these tissues up to this time, the number of nuclei/cells therefore double with each round of cell division. An estimate of the number of cell cycles that occurred in the embryo and endosperm in each 24 hr interval after pollination is thus made from the number of nuclei/cells. The mean cycle time for each 24 hr interval after pollination was calculated from the number of cell cycles that occurred within that period. The mean values were compared with the values estimated by Krishna Rao and Aswani Kumari (1985) in the sib crosses of the interchange homozygote (IP12781) line. The rate of early seed development has been studied in more than 15 inbred lines of pearl millet (Aswani Kumari 1981, Krishna Rao and Aswani Kumari 1981, 1985). The rate of development in the T3-6 interchange homozygote (used as control in this study) was similar to the rate in other normal disomic inbreds studied.

Percent pollen fertility and seed set on open pollinated ears of the interchange heterozygote line was calculated in ten plants. To calculate pollen fertility, anthers ready for dehiscence were used.

Results

In the ears fixed prior to pollination, each of the ovules dissected did have an embryo sac. Visually, (at stereo- and compound microscope level) these embryo sacs were comparable to those in disomic lines (Fig. 2b), they had a well developed egg apparatus with a starch rich egg cell, prominently stained polar nuclei (Fig. 2c, d) and a cluster of antipodal cells. However, seed set in the interchange heterozygote was low. It ranged between 33 to 52% as is expected from the chromosomal aberration. Therefore, in each of the four post pollination samples collected at 24 hr intervals, the 'embryo sacs' were examined to see if any visual indication(s) of functional inadequacy existed.

Fertilization in pearl millet occurs within an hour after pollination and by 24 hap a two celled embryo and a 64 nucleate endosperm are normal (Krishana Rao and Aswani Kumari 1981). Only 174 of the 250 ovules dissected in the 24 hap samples of the interchange heterozygote showed this level of development (Fig. 2e), the rest of the ovules had unfertilized embryo sacs (Table 1). By 48 hap, normal disomics of pearl millet have a ~18 celled embryo and ~600 cells endosperm in most (90-98%) of the ovules (Krishna Rao and Aswani Kumari 1981, 1985). In the T3-6 interchange heterozygote, of the 250 ovules dissected in the 48 hap sample, only 165 showed the expected number of cells in the embryo and endosperm (Fig. 2f). Unfertilized embryo sacs were found in 40 ovules (Table 1). In 45 ovules, the endosperm was still in the free nucleate stage with a fewer number of nuclei than is expected at this stage; the embryo in these ovules, however, had the expected number of cells (Table 2). The developing seed in normal inbreds of pearl millet at 72 hap is reported to have a ~60 celled embryo and a ~2900 celled endosperm and by 96 hap a ~500 celled embryo and a ~5000 celled endosperm are found (Krishna Rao and Aswani Kumari 1981, 1985). Only 50% of the ovules in the 72 and 96 hap samples of the interchange heterozygote showed this level of development (Fig. 2g, h, Tables 1, 2). The other 50% of the ovules in both the samples had either an unfertilized embryo sac or a fewer celled embryo and an endosperm "still in free nucleate condition" with fewer number of nuclei than is expected at these stages of development (Tables 1, 2).

Two kinds of functional disability were thus observed in the ovules from post pollination sam-

Fig. 2. Male and female gametophytes and post pollination developmental stages in the T3-6 interchange heterozygote of pearl millet. a) Pollen grains (male gametophytes), F=fertile, S=binucleate sterile (dp/df). b) Embryo sac (female gametophyte) from the ovule of a floret of an ear at ready to be pollinated stage (pre-pollination stage) a=antipodal cluster, p=polar nuclei. c, d) Micropylar end of the unfertilized embryo sac magnified, e=egg cell, Sy=synergid, p=polar nuclei, en=egg nucleus. e) An embryo sac 24 hap with ~64 nucleate endosperm, a=antipodal cluster. f) An embryo sac at 48 hap with a ~700 celled endosperm a=degenerating antipodal cluster. g) An embryo sac 72 hap with a ~2900 celled endosperm and a globular embryo (→). h) An embryo from a 96 hap sample, i) Micropylar end of the embryo sac magnified showing pollen tube (pt, →) adjacent to the egg cell (e), p=polar nuclei.

Bar=10 μ m.

Table 1. Post pollination development in the T(3-6) interchange heterozygote of pearl millet

hap	% Unfertilized* embryo sacs	Fertilized embryo sacs	
		% with normal Dev.*	% with lag in Dev.*
24	30	70	—
48	16	66	18
72	17	50	33
96	19	51	30

hap=hours after pollination. * Values calculated from observations in 250 ovules, all ovules had a structurally normal looking embryo sac.

Table 2. Rate of endosperm and embryo development in the T(3-6) interchange heterozygote of pearl millet

hap	Type of dev.	Endosperm				Embryo		
		Mean No. of nuclei [§]	No. of cell cycles	Mean cycle time (hr)	Reported cycle time* (hr)	Mean No. of cells [§]	Reported No. of cells*	No. of cell cycles
24	Normal	53.8±5	6	4.1±0.3	4.7±0.13	2	2	1
48	Normal	695±77	9	8.0±0.3	4.6±0.00	19±0.7	17.2±0.9	4
	Lag	248±44	8	12.1±0.43	—	14±6	—	4
72	Normal	2866±673	11	12.0±0.66	11.5±1.2	61±2.8	142.6±17	**
	Lag	540±36	9	24.3±0.32	—	23±4	—	**
96	Normal	4921±463	**	**	**	470±46	279±32	**
	Lag	998±64	10	24.5±0.46	—	112±18	—	**

hap=Hours after pollination. * Values estimated in sib crosses of the T(3-6) interchange homozygote by Krishna Rao and Aswani Kumari (1985). ** Values not calculated because synchrony in cell division is lost. [§] Calculated from 10 ovules.

ples of the interchange heterozygote. 16–30% of the embryo sacs remained unfertilized, 18–33% of the ovules showed a lag in post pollination development.

The unfertilized embryo sacs in the post pollination samples of the interchange heterozygote had a healthy appearance even at 96 hap. They did not degenerate up till this stage. No pollen tube was observed in most of these embryo sacs, only in two of them, a pollen tube was seen (Fig. 2i). In pearl millet, normally a pollen tube enters the embryo sac within 45 min to 1 hr after pollination (Krishna Rao and Aswani Kumari 1981). Thus these embryo sacs failed to attract the pollen tubes and hence remained unfertilized.

The second kind of abnormality observed in the post pollination samples was a slower rate of development. In such ovules a lag in development was first noticeable in 48 hap sample, at this stage it was evident in the endosperm; the embryo, however, had the expected number of cells (Table 2). By 72 hap the development of the embryo also lagged behind, it had fewer number of cells (Table 2). At 96 hap the number of nuclei in the endosperm reached a stage at which cellularization usually takes place. But the endosperm in these ovules (failed to cellularize and) remained free nuclear.

The pollen fertility in the interchange heterozygote line ranged between 36–56% and seed set on open pollinated ears varied between 33–52%.

Discussion

In the T3-6 interchange heterozygote of pearl millet, while breakdown in development of male gametophyte in the ~50% dp/df microspores occurred midway after one mitosis, a normal looking embryo sac was found in all the ovules. However, post pollination, normal development was ob-

served in only about half of them. The others with abnormalities are inferred to be dp/df. The two sexes thus differed in their ability to cope with the genetic lesion: the dp/df microspores could progress only up to midway in male gametophyte development while the dp/df megaspores managed to complete normal female gametophyte development. This difference in the level of tolerance to the dp/df condition between the two sexes could be due to the following reasons.

The microspore expresses its own genes rather than the phenotype of the parent (Walden and Greyson 1986). Intense gene activity has been observed in the pollen (Mascarenhas *et al.* 1986, Sari Gorla *et al.* 1986, Mulcahy 1987). Therefore, it is possible that microspores with dp/df chromosomal condition cannot develop normally. The development of the female gametophyte could however be directed by the maternal genome. Studies of the female gametophyte in the autotetraploids and a desynaptic mutant in pearl millet point towards this conclusion (Koduru and Krishna Rao 1978, Aswani Kumari 1981, Murthy 1982). Apparently, a supportive role for the maternal tissue is "normal". In both the autotetraploid and the desynaptic mutant, the percent pollen fertility correlated with percent seed set. In the autotetraploid pearl millet, while ~20% microspores with aneuploid condition did not initiate male gametophyte development and appeared as sterile pollen (Koduru and Krishna Rao 1978), ~95% of the ovules possessed normal looking embryo sacs (Krishna Rao and Aswani Kumari 1980). In the monofactorially inherited desynaptic mutant of pearl millet (showing extreme desynapsis), only 8% of the pollen looked normal fertile (Murthy 1982), whereas 78–83% of the ovules had normal looking embryo sacs (Aswani Kumari 1981). Similar is the situation in the T3–6 interchange heterozygote of pearl millet described here.

The higher level of tolerance to chromosomal aberrations on the female side than in the male counterpart could also be due to the absence of competition from (mega-) spores with normal chromosome complement in the ovule (Sybenga 1992). There is only one megaspore mother cell in the ovule and when the interchange multivalent shows adjacent or indifferent orientation, all the resultant megaspores are dp/df. The dp/df megaspores in the absence of competition from normal spores could have utilised the nutrients from the maternal tissue and developed into normal looking embryo sacs. The dp/df microspores on the other hand have an approximately equal number of microspores with normal chromosome complement to compete with (Janse 1987). "Functionally less competent dp/df or aneuploid male gametophytes" in some chromosomal interchanges and trisomics of maize and rye were found to succeed in fertilization when, artificially, the chromosomally balanced or euploid fertile pollen was excluded (Walden and Greyson 1986, Janse 1987, Niizeki and Saito 1988). In *Arabidopsis thaliana* the dp/df megaspores of a translocation (involving chromosomes 2 and 5) heterozygote failed to develop into embryo sacs. In the dp/df megaspores either a cell division was not initiated or development stopped after one division. They remained uni- or bi-nucleate (Ray *et al.* 1997). Whether they represent the two kinds of dp/df spores is not mentioned by Ray *et al.* (1997). The dp/df region in these spores must have had genes controlling cell division/development.

The two kinds of dp/df spores could not be distinguished on the male side in the T3–6 interchange heterozygote of pearl millet. Both kinds of microspores degenerated at binucleate stage. On the female side, in the dp/df products, functional incompetence was visible at two different-gametic and zygotic (embryonic) stages. Some embryo sacs failed to get fertilized (gametic lethality), some succeeded in fertilization but the embryo degenerated (apparently) due to the dp/df condition (zygotic lethality). These two kinds might represent the embryo sacs with the different dp/df conditions. Differences in the two kinds dp/df products have been reported on the male side in some interchanges of maize and *Allium triquetrum* (Burnham 1962, Rickards 1983, Walden and Greyson 1986). We have found such differences (Table 1) on the female side in the T3–6 interchange heterozygote of pearl millet.

In the dp/df embryo sacs that failed to attract the pollen tube (and therefore not succeed in fertilization) the genetic lesion could have affected their ability to elicit a chemotropic signal which di-

rects the pollen tube into them. Control of pollen tube growth by the embryo sacs was first reported in avocado (Sedgley 1978). In an interchange heterozygote of *Arabidopsis thaliana* the ovules with no embryo sac did not have a pollen tube (Ray *et al.* 1997). Ray *et al.* (1997) concluded that the pollen tube is guided by the female gametophyte. The synergids are assumed to synthesise chemotropic substances (Went and Willemse 1986). A calcium gradient formed by synergids has also been implicated in the chemotropic signal (Chaubal and Reger 1990, Reger *et al.* 1992). A considerable number of the normal looking embryo sacs in the desynaptic mutant and autotetraploid of pearl millet (aneuploid for different chromosomes) also failed to get fertilized when pollinated (Aswani Kumari 1981, Krishna Rao and Aswani Kumari 1981). Thus the genetic elements governing the chemotropic function of the embryo sac may be distributed on several chromosomes.

The dp/df embryo sacs that succeeded in fertilization did not achieve a normal rate of development. The triploid endosperm was the first to be affected, its nuclei have two sets of dp/df chromosomes. The embryo coped for a longer while; its nuclei have one set of dp/df chromosomes. Eventually both degenerated. Degeneration of the embryo during early stages of seed development has also been observed in some ovules in the desynaptic mutant and autotetraploid of pearl millet (Aswani Kumari 1981, Krishna Rao and Aswani Kumari 1980). The early development of the embryo is believed to be directed by the gene activity in the egg (Raghavan and Sharma 1995). Intense gene activity as evidenced by presence of labelled mRNA has been reported in the egg cell (Raghavan 1990, Bimal *et al.* 1994, Bimal and Willemse 1996). Thus the chromosomally unbalanced egg could not sustain normal development of the zygote. At present there is little data available about the regulation of ovule and embryo sac development and the temporal and spatial patterns of gene expression in the embryo sac (Reiser and Fisher 1984). For example the extent to which the genetic programmes overlap between the male and female gametophytes on one hand and between the female gametophyte and sporophyte on the other is not known. Also unknown is the time when actually the gametophyte specific genes are expressed during the development of the female gametophyte (Reiser and Fisher 1984). The molecular genetic approaches required to address some of these questions could be done using this easy technique of isolation of embryo sacs and the cytogenetic mutants as the one described here.

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