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An Asynaptic Mutation in Soybean (*Glycine max* (L.) Merrill) Associated with Total Absence of Sister Chromatid Cohesiveness

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Summary A mutation affecting microsporogenesis and causing complete male sterility has been detected in line BR97-13777H from Embrapa National Soybean Research Center breeding program. In prophase I, the meiotic behavior of this mutant was similar to the synaptic mutants previously reported in soybeans. Many univalents and few bivalents were found in diakinesis. The main and distinctive characteristic of this mutant was the complete inability of univalents to congregate in the metaphase plate. Scattered in the cytoplasm, univalents underwent premature sister chromatid separation, so that 80 chromatids could be easily counted. Telophases II with a varied number of different-sized nuclei were observed. Pollen sterility was estimated at 93.12%. The inability of univalents to congregate at the equator made us classify it as asynaptic mutant. Its special characteristic related to precocious sister chromatids separation suggests that it is probably defective in proteins that promote sister chromatid cohesion. Segregation ratio for male sterility was 3 : 1 and shows that mutation, similar to other synaptic mutations previously reported in soybean, is associated with the homozygous condition of a single recessive gene. Allele tests with the *st* series of synaptic mutants are in progress.

Key words Soybean *Glycine max*, Microsporogenesis, Asynaptic mutation, Sister chromatid cohesiveness, Pollen viability.

Meiosis results in halving the chromosome complement, a process required for the formation of haploid gametes or cells. This involves a complex orchestration of events including chromosome pairing at zygotene, genetic exchange at pachytene, chiasmata formation at diplotene and chromosome segregation at anaphase I and II (Franklin *et al.* 1999). Synaptic mutants in higher plants provide excellent tools for studying genetic control of chromosome pairing, synaptonemal complex assembly, and chiasma formation during meiotic prophase I.

A great number of *as* and *ds* genes has been reported in higher plants (Gottschalk and Kaul 1980a, b, Koduru and Rao 1981). They control the homologous chromosome pairing and the chiasmata frequency respectively. The *as* genes cause asynapsis, *i.e.*, the failure of chromosome pairing (Randolph 1928), whereas *ds* genes cause desynapsis, *i.e.*, a pronounced reduction of chiasma frequency or complete lack of chiasma formation (Li *et al.* 1945). Instead of bivalents, univalents are present in the latter stages of the first meiotic prophase and in metaphase. The delimitation of these 2 gene groups is possible only by studying the pachytene phase. Due to methodological limitations studies on pachytene pairing are not always feasible in certain species, particularly in mutant plants. In many instances such studies have been left undone, leading to much confusion in the literature concerning the 2 phenomena (Gottschalk 1987). Since such distinction is not always possible, as some species are not amenable to analysis at pachytene, Riley and Law (1965) suggest that "synap-

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tic mutant" is a better alternative term to describe the lack of chromosome pairing at prophase I.

Several soybean male-sterile and female-sterile lines, called *st1*, *st2*, *st3*, *st4*, *st5*, *st6*, *st7* and *st8* have been described genetically and cytologically (Owen 1928, Hadley and Starnes 1964, Palmer 1974a, b, Winger *et al.* 1977, Palmer and Kilen 1987, Skorupska and Palmer 1990, Ilarslan *et al.* 1997, Palmer and Horner 2000). All of them have been cytologically characterized as asynaptic or desynaptic mutants and inherited as single recessive genes. In this paper, the cytological details are provided of a spontaneous male-sterile mutation found in soybean line BR97-17333H selected in the breeding program developed by Embrapa-National Soybean Research Center.

Materials and methods

Soybean plants segregating for the male-sterile trait (MS) have been detected in the Embrapa Soybean breeding program. The male-sterile plants could not be distinguished from the fertile ones until production of pods, when total absence or only a few pods were observed on green plants that retained their leaves on maturity. In 1996 we observed a plant progeny-row segregating for an apparent male-sterile character. This spontaneous mutant line was an F4-derived plant from cross RS 7×[BR16(4)×IAC 12]. Male-fertile plants from this progeny-row were selected and evaluated for use in the following year. They were called BR97-13777. Within segregating families, a 3 : 1 ratio has been reported which indicated that sterility was caused by a single recessive gene. Trait is maintained in the heterozygous form in the Soybean Germplasm Collection at Embrapa Soybean, Londrina PR Brazil. Plant progeny tests were conducted in a greenhouse so that heterozygous plants that would segregate for sterility could be identified. About 12 seeds of normal plants were grown in pots. Progenies with fertile and male-sterile plants were considered heterozygote. Remaining seeds from the MS heterozygotes were once more put in pots and left to grow. First flowers were tested for pollen viability using 0.5% propionic carmine for staining. The sterile plants were identified and flower buds collected in ideal stage for meiotic analysis between 9 : 00 and 12 : 00 a.m. and fixed in a mixture of ethanol 95%, chloroform and propionic acid (6 : 3 : 2 v/v) for 24 h, after which they were transferred to 70% alcohol and stored at 4°C until use. Microsporocytes were prepared by squashing and stained with 1% propionic carmine. Ten male-sterile plants were cytologicaly analyzed. The number of microsporocytes analyzed per plant ranged from 289 to 491, comprising diakinesis to tetrad stages. A number of 1000 pollen grains were scored at random in each plant to estimate pollen viability. Plump, well-stained pollen grains were counted as viable.

Results

Light-microscope reports of microsporogenesis from mutant and fertile plants were made. Unfortunately, the earlier meiotic stages were not amenable to accurate cytological analysis. Consequently only meiotic figures from diakinesis to tetrad stage were analyzed from the squash preparations. Major differences in chromosome behavior between fertile and sterile plants could be detected at diakenesis. Twenty bivalents with 1 or 2 terminal chiasmata were normally observed in fertile plants (Fig. 1a), while few bivalents and a high and variable number of univalents in sterile plants were frequently found (Fig. 1d). Meiosis in fertile plants progressed normally with 20 bivalents congregating at metaphase plate in metaphase I (Fig. 1b) and segregating to the poles at anaphase I (Fig. 1c) as well as in the second division. The meiotic product was a tetrad of 4 equally-sized microspores. In sterile plants diakinesis showed several univalents and few bivalents with 1 or 2 terminal chiasmata. Chiasmata underwent precocious terminalization and 40 univalents were found after diakinesis. The mutant's main and distinctive characteristic was the inability of the univalent chromosomes to congregate in the metaphase plate, so that typical figures of metaphase I were never observed. Instead of typical metaphase I and anaphase I, the microsporocytes exhibited widely scat-

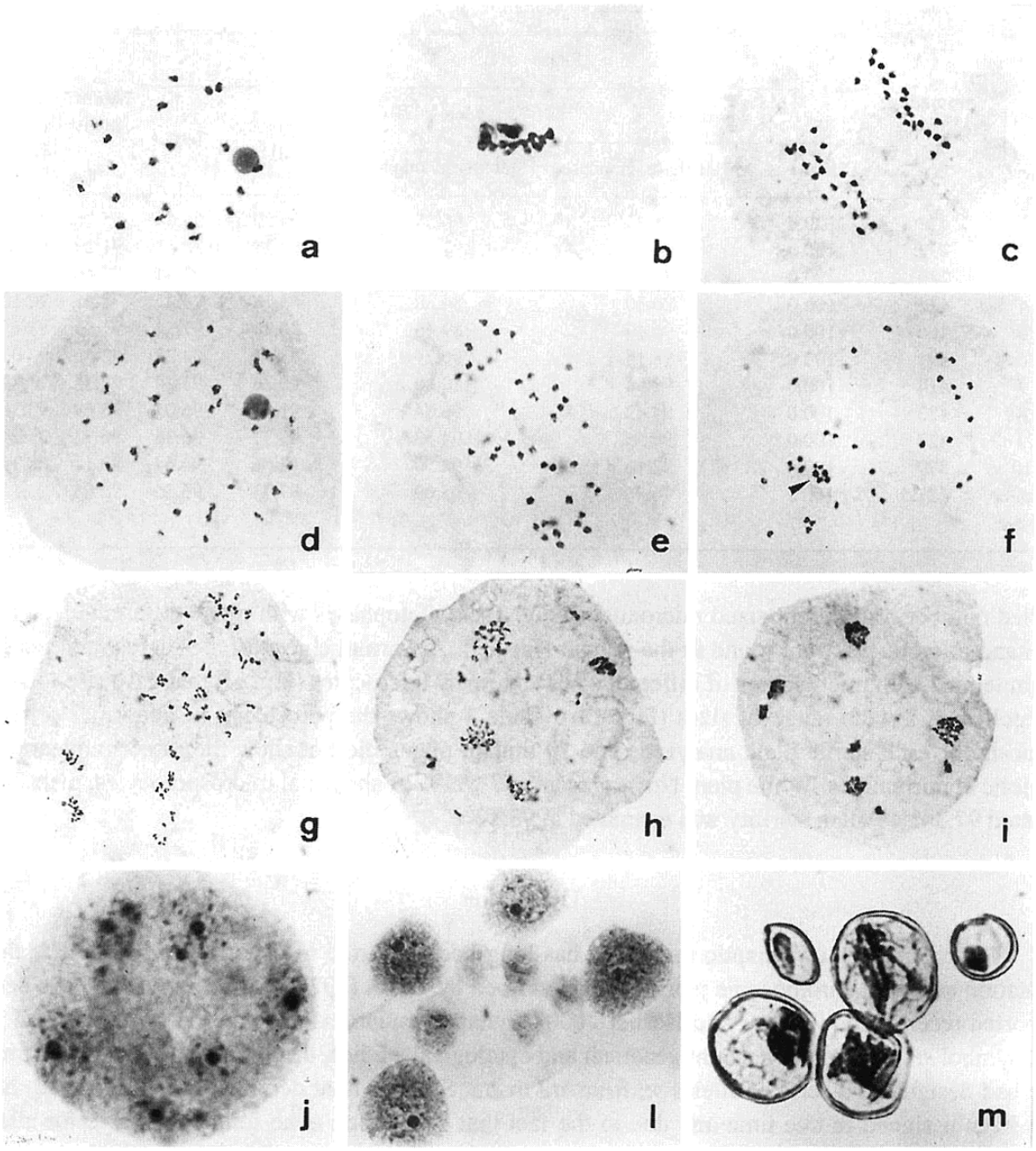

Fig. 1. Some aspects of chromosomal behavior in the line BR93-13777H. a–c) Diakinesis (a), metaphase I (b), and anaphase I (c) in fertile plants. Note the normal aspects of each phase. d) Diakinesis showing 9 bivalents and 22 univalents in a sterile plant. e–g) Metaphase I/anaphase I with 40 univalents scattered in the cytoplasm. Note that some of them show tendency for agrouping (arrowheads). g) No typical anaphases II showing total sister chromatid separation. Note the presence of 80 chromatids, and the proximity of them to form several micronuclei of different size. h–j) Different stages of telophases II. Note the presence of several nuclei and micronuclei of different sizes. l) Tetrad with many microcytes of different sizes. m) Sterile pollen grains not equally sized.

tered univalents in the cytoplasm (Fig. 1e, f). Microsporocytes were grouped according to chromosome proximity, albeit with high condensation (Fig. 1f). Nevertheless, typical telophases I were never observed. Similar to second division stages, each group of chromosomes, set in the place in which it was formed, progressed in meiosis through sister chromatid separation. Thus, 80 chromatids could be easily counted in the microsporocytes (Fig. 1g). Typical metaphase II and anaphase II were not observed. The 80 chromatids, also depending on their proximity, were organized into a

Table 1. Percentage of cells with meiotic abnormalities in each phase of meiosis and pollen sterility

Plant	Number of cells/plant	Phases of meiosis					Means/ plant	Pollen sterility
		Meiosis I		Meiosis II	Telophase II	Tetrad		
		Diakinesis	Metaphase/anaphase	Metaphase/anaphase				
1	472	100.0	98.95	96.58	94.20	95.65	95.76	96.14
2	432	100.0	96.28	91.06	87.27	88.61	91.20	88.66
3	289	100.0	99.37	94.87	94.92	93.84	94.46	99.50
4	422	100.0	90.30	84.30	68.18	78.82	78.91	90.10
5	466	100.0	96.49	89.46	80.26	97.59	91.85	88.15
6	491	100.0	98.15	90.79	83.53	93.75	92.46	93.07
7	414	100.0	98.96	100.0	90.28	93.24	97.34	98.87
8	423	100.0	94.42	96.84	91.30	96.05	96.69	93.36
9	427	100.0	99.55	94.52	95.31	96.36	96.72	95.24
10	379	100.0	92.16	92.50	92.06	94.64	93.14	88.15
Means/ phase	421.5	100.0	96.46	93.09	87.73	96.86	92.85	93.12

varied number of different-sized micronuclei (Fig. 1h, i). Telophases with many different-sized nuclei and micronuclei were found in the mutant (Fig. 1j). Abnormal chromosome segregation resulted in tetrads with microspores of different sizes and many microcytes (Fig. 1l) that gave rise to sterile pollen of a wide range of sizes (Fig. 1m). Table 1 shows the percentage of abnormal cells in meiosis for each sterile plant analyzed. The 10 mutant plants did not show the same frequency of meiotic abnormalities. While plant No. 4 presented 78.91% of abnormal microsporocytes, plant No. 7 rated 97.34%. Pollen sterility was estimated at 93.12%.

Discussion

The occurrence of synaptic mutations has long been reported to occur in soybeans. The first mutation affecting chromosome pairing was described by Owen (1928) and new mutants have been reported recently too (Palmer and Horner 2000). Synaptic mutations in soybeans are designated by the symbol *st*. Taking into account genetical and cytological studies, the Soybean Genetics Committee had designated a serie of alleles *st*, from *st2* to *st8*. Since mutant decribed by Owen (1928) had not been assigned in due time and due to the fact that gene stock is no longer available for allele tests, Hadley and Starnes (1964) suggested to name this mutation *st1*. Although cytological details were not described for *st1* sterile plants, Hadley and Starnes (1964) found that plants resembled asynaptic/desynaptic plants assigned *st2* and *st3*. Mutants *st4* (Palmer 1974a), *st5* (Palmer and Kaul 1983) and *st6st7* (Ilarslan *et al.* 1997) were classified as desynaptic according to their cytological basis. *st8* (Palmer and Horner 2000) was classified as a synaptic mutant.

There is a common opinion among cytologists that it is really dificult to distinguish asynaptic from dysynaptic mutations since studies of pachytene chromosome pairing is not always amenable in some species. Studies on synaptic soybean mutants described the cytological data as follow: (a) *st2* and *st3* showed many synapsed pachytene chromosomes, albeit at diakinesis and metaphase I most chromosomes occurred as univalents; (b) *st4* showed only terminal desynapsis in some pachytene chromosomes and a few microsporocytes, while complete lack of pairing was observed at diakinesis; (c) *st5* had the same cytological behavior as that of *st4*; (d) *st6* and *st7* were stained with mithramycin; thus, cytological details of prophase I were not provided; due to the segregational behavior at anaphase I and II, sterility was attributed to desynapsis; (e) *st8* exhibited almost complete absence of chromosome pairing, but no picture of meiotic stages has been provided. Results

from *st4* to *st8* were obtained by Palmer and his group at Iowa State University. They always emphasized difficulties in observing earlier meiotic stages and in determining whether low level desynapsis was caused by initial absence of chromosome pairing or by post-synapsis dissociation (Palmer and Kaul 1983). For these reasons, Palmer and Horner (2000) preferred to classify *st8* a synaptic mutant.

Because of the chromosomes' thin and tangled shape, prophase I preparations from zygotene to diplotene did not give good pictures to pinpoint where the synapsis process was lacking in the mutant. Nevertheless, one special cytogenetic characteristic discriminated asynapsis from desynapsis. Similar to other synaptic soybean mutants, the chromosomes appear as univalent at diakinesis. However, while in soybean mutants the univalents showed ability to congregate in the metaphase I plate, our mutant did not show this characteristic, and the 40 univalents remained widely scattered in the cytoplasm. According to Peirson *et al.* (1997), asynaptic mutants can be differentiated from desynaptic ones by the ability of univalents to congregate in metaphase I plate. In asynaptic mutants, the univalents are randomly scattered throughout the cytoplasm and never align at the equator plate. Our mutant may thus be considerated asynaptic. Since synaptonemal complex formation could not be observed at this level of analysis, further studies are required (*e.g.* chromosome spreads and electron microscopy) in order to ascertain the above. Behavior of univalents is due to the fact that they have only 1 centromere, so that they are unable to maintain the normal bipolar orientation along the metaphase I spindle; therefore, univalents move independently to the poles (Peirson *et al.* 1997). The formation of a chiasma in normal cells provides a connection between homologues that restrain a poleward movement and cause tension at the kinetochores. In the absence of a chiasma, the resulting univalent kinetochores do not feel any tension and produce a signal that causes the cell to delay anaphase (Dawe 1998). Among the factors thought to sense tension at the kinetochores in *Drosophila melanogaster* there is a protein called ZW10 (Williams 1996, Allshire 1997). An *Arabidopsis* homologous of the *zw10* gene has been identified (Starr *et al.* 1997), suggesting that the tension-sensing mechanisms, similar to those described in animals, could operate in plants too.

Asynapsis in the mutant was not total because some bivalents could be seen in some cells at diakinesis. However, these bivalents did not maintain their terminal chiasmata until metaphase I. Defects in synapsis not only resulted in recombination and chiasmata deficiences, but might also hinder establishment of sister chromatid cohesion necessary for proper chromosome segregation (Bickel and Orr-Weaver 1996). The presence or absence of sister-chromatid cohesion marks important events in the cell cycle, but the chromatid separation process remains mysterious (Nasmyth *et al.* 2000). According to Miyazaki and Orr-Weaver (1994), the metaphase/anaphase transition in meiosis I is correlated with a loss of sister chromatid arm cohesion. The sequential release of cohesion in meiotic chromosomes first between the chromosome arms at meiosis I, and then between sister centromeres at meiosis II, hints at the possible underlying complexity of the mechanisms and regulation of such cohesion. Synaptic mutants have been used as a powerful tool to understand the sister chromatid cohesion. Mutations in protein-encoding genes required for sister chromatid cohesion should result in the premature separation of sister chromatids prior to metaphase/anaphase transition. In these mutants, the univalents often undergo sister chromatids separation during anaphase I, and the number of individual chromatids recorded at meiosis I is more than the diploid number of chromosomes. Although present mutant maintained the cohesion of the chromatids in the univalent, so that the diploid soybean chromosome number ($2n=40$) could be seen, the sequential behavior was extremely particular when compared with the other *st* synaptic mutants in this species. Although in the metaphase and anaphase stages chromosomes were not placed in typical metaphase plates, *i.e.* chromosomes were scattered in the cytoplasm, the cohesion of sister chromatids was totally released and 80 chromatids could be easily counted. Such differential characteristic of our mutant, when compared with the *st* series, suggests that it is probably defective in sister

chromatid cohesion. Isolation of mutations provides a powerful tool for identifying and characterizing functions that control sister chromatid cohesion. Further detailed meiotic studies involving the synaptonemal complex and/or cohesion between sister chromatids could be of some help to understand better and explain the causes of this abnormality.

Univalent chromosomes in synaptic mutants are randomly distributed to the daughter nuclei or lost as micronuclei in the cytoplasm. The unequal distribution of chromosomes in the first and second anaphases results in the production of microspores of various sizes and ploidy numbers, that normally do not develop into viable pollen grains. Concerning the present mutant, the abnormal chromosome segregation in both divisions resulted in high pollen sterility (93.12%). From *st2* to *st8*, the synaptic mutants reported in soybeans always showed over 90% of sterile pollen grains. Gametes with the complete genome are rarely produced by asynaptic or desynaptic mutants and result in almost completely male and female sterile plants (Palmer and Heer 1976). Owing to the fact that most synaptic mutants cause female sterility too, Peirson *et al.* (1997) suggested that a set of genes controls homologous chromosome pairing and recombination in anthers and ovules. All *st* series of synaptic mutants reported in soybeans were also male-sterile and female-sterile. Unfortunately, we lack information on female viability of line BR97-13777H.

Although causing male and female sterility, one of the major consequences of synaptic mutations is the gametic unbalance which may lead to various forms of aneuploidy in later generations (Khush 1973). In soybeans, aneuploids have been reported among the progeny of synaptic mutants (Hadley and Hymowitz 1973, Palmer 1974a, b). The *st2* and *st3* soybean mutants produced most of the progeny tetraploid (Palmer 1974b), while the *st4* had more aneuploids among its progeny (Palmer and Heer 1976). On the other hand, *st5* with chromosome abnormalities similar to those of *st4*, failed to generate more self- or cross-pollinated seed. Although we did not test the capacity of our mutant to generate aneuploids, the high frequency of meiotic abnormalities suggests that it will not be suitable for this purpose.

Segregation ratio in BR97-13777H, where the mutation for male sterility originally appeared, was 3 : 1, and remained constant in the progenies of heterozygotes fertile plants after selfing. Thus, this mutation is caused by a single recessive gene. Allele tests of mutant with some of the *st* soybean mutant series available in the Soybean Genetic Type Collection (USDA/ARS) are in progress. Some phenotypic characteristics of the BR97-13777H mutant are similar to those of *st2* and *st3* asynaptic/desynaptic mutant (Hadley and Starnes 1964) and *st4* desynaptic mutant (Palmer 1974a). Late in the season, fertile plants were mature, but the sterile ones were green and vigorous. Since their cytological characteristics are very different from mutant, a new mutation has been suggested.

The identification of new synaptic mutants supplements increasing data on the *st* genetic mutant series and provide useful cytological and genetics information about the manner male sterility occurs in higher plants. Male sterility is considered a powerful tool in hybridization breeding program. According to Jin *et al.* (1998), the major obstacle in F_1 hybrid soybean seed production is the intensive hand-labor requirements for large number of pollinations. However, there are several limitations in the use of nuclear male sterility for hybrid seed production due to the fact that this type of sterility is predominantly determined by recessive alleles, while only the homozygous recessive may be identified in segregating populations.

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