Bilateral sexual tetraploidization in red clover[1]

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Received July 1, 1984
Revised manuscript received October 19, 1984

PARROTT, W. A., R. R. SMITH, and M. M. SMITH. 1985. Bilateral sexual tetraploidization in red clover. Can. J. Genet. Cytol. **27**: 64–68.

Twenty-four diploid red clover (*Trifolium pratense* L.) plants, from the cultivars 'Arlington', 'Florex', and 'Redman', and C760, a Wisconsin synthetic, were tested for $2n$ egg production by crossing them with tetraploid plants. Four plants were identified and selected as $2n$ egg producers. These plants were then crossed with a known producer of $2n$ pollen in an attempt to obtain tetraploid plants through bilateral sexual polyploidization. Thirteen percent of the seed obtained in one of the $2x-2x$ crosses were tetraploid. The female plant produced an estimated 0.14 $2n$ eggs per 1000 flowers in the cross. In a second experiment, bilateral sexual polyploidization was attempted, using 14 plants which had not been previously tested for $2n$ egg production. Two of these plants produced one and two tetraploids each, representing $2n$ egg frequencies of 3.33 and 5.00 $2n$ eggs per 1000 flowers, respectively. It seems likely that it will be possible to use bilateral sexual polyploidization to tetraploidize red clover germplasm in the future.

Key words: tetraploid red clover, *Trifolium pratense*, $2n$ gametes.

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Vingt-quatre plantes de trèfle rouge diploïde (*Trifolium pratense* L.) provenant des cultivars 'Arlington', 'Florex' et 'Redman', ainsi que du cultivar synthétique du Wisconsin, C760, ont été croisées avec des plantes tétraploïdes en vue de la production d'oosphères $2n$. Quatre plantes ont été identifiées et sélectionnées comme productrices d'oosphères $2n$. Ces plantes ont été croisées avec une productrice connue de pollen $2n$ dans une tentative d'obtenir des plantes tétraploïdes au moyen d'une polyploïdisation sexuelle bilatérale. Treize pour cent des graines obtenues chez l'un des croisements $2x-2x$ furent tétraploïdes. La plante femelle a produit une moyenne de 0,14 oosphères $2n$ par 1000 fleurs, suite au croisement. Dans une seconde expérience, un essai de polyploïdisation sexuelle bilatérale a été poursuivi avec 14 plantes qui n'avaient pas été soumises à une vérification quant à leur production d'oosphères $2n$. Deux de ces plantes ont produit respectivement une et deux tétraploïdes, ce qui représentait des fréquences respectives de 3,33 et de 5,00 oosphères $2n$ par 1000 fleurs. Il semble donc qu'il sera désormais possible de recourir à la polyploïdisation sexuelle bilatérale pour obtenir des cellules germinales tétraploïdes chez le trèfle rouge.

Mots clés: trèfle rouge tétraploïde, *Trifolium pratense*, gamètes $2n$.

[Traduit par le journal]

Introduction

Bilateral sexual polyploidization (BSP) can be defined as the mating of male and female $2n$ gametes (gametes with the chromosome number of the sporophytic generation) to obtain polyploid progeny (Mendiburu and Peloquin 1976). Harlan and deWet (1975) suggested that polyploids in the angiosperms originated primarily from the union of $2n$ gametes, rather than through spontaneous doubling of somatic chromosomes. deWet (1979) has proposed that polyploids are formed when a haploid gamete mates with a $2n$ gamete to produce a triploid. Then, a $2n$ gamete from the triploid mates with another haploid gamete to produce a tetraploid. However, others have considered BSP to be a more likely alternative (den Nijs and Peloquin 1977*a*, 1977*b*; Bingham and McCoy 1979), as triploids occur at a very low frequency in many angiosperms. Furthermore, once the initial tetraploid is formed through BSP, it could continue to mate with

[1]Cooperative investigation of USDA-ARS and the Wisconsin Agricultural Experiment Station, Madison, WI, U.S.A. 53706. This research was supported in part by U.S. competitive grant No. 8100349.

TABLE 1. Number of tetraploid red clover plants produced and frequency of $2n$ egg production from $2x-4x$ crosses

Female parent[a]	No. of heads harvested	Total no. of seeds	No. of seeds planted	No. of $4x$ plants produced	% $4x$ seed[b]	No. of $2n$ eggs per 1000[c]
Confirmed crosses[d]						
Arl-A	715	77	30	6	20	0.215
Arl-E	98	8	8	0	0	0.000
C760-A	12	12	0	0	0	0.000
C760-C	140	8	8	0	0	0.000
C760-E	200	56	30	5	17	0.476
Flor-A	941	1637[e]	30	0	0	0.000
Flor-C	115	2	2	0	0	0.000
Flor-E	260	311	30	0	0	0.000
Red-A	377	16	16	0	0	0.000
Red-D	309	21	21	0	0	0.000
Red-E	215	101	30	1	3	0.141
Red-F	335	39	30	0	0	0.000
Unconfirmed crosses[d]						
Arl-B	394	12	9	1	11	0.033
Arl-C	793	3608[e]	30	2	7	3.185
Arl-D	390	330	30	21	70	5.923
Arl-F	306	30	30	21	70	0.686
C760-B	494	29	29	11	40	0.235
C760-D	34	11	11	0	0	0.000
C760-F	63	2	2	0	0	0.000
Flor-B	544	122	30	3	10	0.224
Flor-D	102	35	30	19	63	2.161
Flor-F	505	81	30	11	37	0.593
Red-B	212	56	30	6	20	0.528
Red-C	520	200	30	11	37	1.423

[a]Arl, 'Arlington'; Flor, 'Florex'; Red, 'Redman'.
[b]% $4x$ seed = (No. of $4x$ plants produced)/(No. of seed planted) × 100.
[c]Estimated on basis of 100 flowers/head.
[d]Confirmed = positive identification of hybrid seed possible; unconfirmed = positive identification of hybrid seed not possible.
[e]Estimated on basis of one seed = 2 mg.

diploid individuals which produce $2n$ gametes in the original population, producing more tetraploid individuals, in a continuing process referred to as unilateral sexual polyploidization (USP) (Mendiburu and Peloquin 1976).

Tetraploids have been obtained by BSP in potato, *Solanum tuberosum* L. (Mendiburu and Peloquin 1977), and BSP has been proposed as a breeding technique for potatoes because of the high tuber yields of tetraploid progeny obtained via BSP (S. J. Peloquin).[2] When a diploid alfalfa (*Medicago sativa* L.) population was extracted from cultivated tetraploids, tetraploid plants apparently resulted from BSP in subsequent sexual generations. Consequently, plants producing $2n$ gametes had to be removed to prevent the population from reverting to the tetraploid level (Bingham and McCoy 1979).

Existence of $2n$ eggs in red clover (*Trifolium pratense* L.) was reported in 1974 (Ellerström and Sjödin 1974; Anderson and Taylor 1974). Since that time, there has been interest in using $2n$ eggs to obtain tetraploid red clover. Systematic searches for $2n$ eggs in red clover by Broda and Smith (1980) and by Taylor and Giri (1983) revealed that plants with useful levels of $2n$ egg production exist. The discovery of functional $2n$ pollen in red clover (Parrott and Smith 1984) suggested that BSP may be an alternative to the use of chemical agents to obtain tetraploid clover. As some types of $2n$ gametes can be heterozygous, developing polyploids sexually can avoid the inbreeding depression that is inherent with use of chemical agents to double chromosome numbers. The production of chimeras is also prevented.

The current objective was to identify red clover

[2]Peloquin, S. J. 1982. New approaches to breeding in the potato of the year 2000. International Potato Center. Tenth Anniversary Congress.

TABLE 2. Number of tetraploid red clover plants produced and frequency of $2n$ egg production from $2x-2x$ crosses

Female parent[a]	No. of heads harvested	Total no. of seeds	No. of seeds planted	No. of $4x$ plants produced	% $4x$ seed[b]	No. of $2n$ eggs per 1000[c]
Experiment 1						
Arl-A	551	57	30	4	13	0.138
Arl-D	313	305	30	0	0	0.000
C760-B	184	13	13	0	0	0.000
Flor-F	397	1039	30	0	0	0.000
Experiment 2						
LP-4	3	1	1	1	100	3.333
LP-10	4	2	2	2	100	5.000

[a]Arl, 'Arlington'; Flor, 'Florex'; LP, long petiolule.
[b]% $4x$ seed = (No. of $4x$ plants produced)/(No. of seed planted) × 100.
[c]Estimated on basis of 100 flowers/head.

plants that produce $2n$ eggs on a regular basis, and which could be used for either bilateral or unilateral sexual tetraploidization.

Materials and methods

Isolation of 2n *egg producers*

Twenty-four diploid ($2n = 2x = 14$) plants were tested for $2n$ egg production by using them in $2x-4x$ crosses. The 24 plants were six each from the cultivars 'Arlington', 'Florex', and 'Redman', and six from C760, a Wisconsin synthetic. Three to six ramets were obtained from each plant to sample as many flowers as possible. Ramets from each plant were transplanted into separate bee cages at Arlington, Wisconsin, during the 1982 growing season. Four tetraploid plants (developed by Dr. N. L. Taylor, University of Kentucky) were placed in each cage to serve as a pollen source. Therefore each cage contained four tetraploid plants and the ramets of one $2x$ plant. Honey bees (*Apis mellifera* L.) were used as pollinators.

Seed heads were harvested only from the diploid plants. Tetraploids obtained from these would be the result of the union of $2n = 2x$ eggs of the diploid parent and $n = 2x$ pollen from the tetraploid plants (i.e., USP). Because diploid plants are self-incompatible, little if any seed was expected as the result of selfing on the diploid. In addition the leaf mark characteristic (a dominant genetic marker) present on one parent was used as an assurance against mechanical mixture of seed during harvest. Leaf marks also helped differentiate between hybrid and selfed progeny. Preliminary identification of the progeny ploidy level was made on the basis of pollen morphology (Taylor et al. 1976) and verified with chromosome counts in root-tip cells, as described by Giri et al. (1981).

Bilateral sexual polyploidization

Four plants with high frequencies of $2n$ egg production were identified in 1982. The four plants were 'Arlington'-A, 'Arlington'-D, C760-B, and 'Florex'-F. These were used in a BSP attempt, referred to as experiment 1, during the 1983 growing season. Three to four ramets from each plant were transplanted into respective pollination cages at Arlington, Wisconsin, together with three ramets of a pollen parent, a diploid plant from the cultivar 'Chesapeake', designated as C51. This plant is a synaptic mutant and produces $2n$ pollen exclusively. It has been described previously (Parrott and Smith 1984). The ploidy of 20–30 progeny from each female parent plant was identified as previously described except that chromosomes in root-tip cells were stained with modified carbolfuchsin (Kao 1975).

In a second experiment, an attempt was made to obtain tetraploids through BSP, without prior selection of the female parents for $2n$ egg production. Fourteen highly male sterile genotypes were crossed to C51 (pollen parent) in a pollination cage in the greenhouse, using honey bees to effect pollination. These 14 male sterile plants were also homozygous for long petiolules, a recessive genetic marker (M. M. Smith and R. R. Smith, manuscript in preparation).

Results

Results from the search for $2n$ eggs are summarized in Table 1. In 12 crosses positive identification of the tetraploids could not be completed because of the incomplete expression of the genetic marker. Three plants from this category were included in subsequent $2x-2x$ matings.

No seeds were obtained in 9 of the 12 crosses where positive identification of hybrid progeny should have been possible. From the three remaining crosses, 20, 17, and 3%, respectively, of seed harvested and tested was hybrid. Assuming that each tetraploid seed obtained represented one $2n$ egg and that each head produced 100 flowers, the estimated frequency of $2n$ eggs per 1000 was 0.215, 0.467, and 0.157, respectively.

When the progeny of the $2x-2x$ crosses made in 1983 were examined cytologically, four tetraploid plants and one triploid plant were identified from the cross 'Arlington'-A × C51. No polyploid progeny were identified from the remaining three crosses (Table 2). The female parents that produced no $2n$ eggs in the $2x-2x$ crosses were also the three parents that were selected out of the unconfirmed category (Table 1). This might explain why they produced no $2n$ eggs in 1983.

Fifty-seven seeds were obtained from the cross 'Arlington'-A × C51. Thirty of these seeds were tested for ploidy, and four proved to be tetraploid. Thus, 13% of the seed were tetraploid. This represents a frequency of 0.138 $2n$ eggs per 1000 flowers. Only one triploid progeny resulted from all the crosses.

Ten of fourteen plants used as female parents in the second experiment failed to produce any seed. Two plants produced progeny with long petiolules (indicating they resulted from crosses among the plants used as female parents). These two plants also produced six diploid plants with normal petiolules. These probably resulted from haploid (n) pollen produced by C51 (being a synaptic mutant, only $(1/2)^7$, or 1 in 128 PMCs, will form haploid pollen. Tetraploid progeny were obtained from the remaining two plants (Table 2). One genotype gave one tetraploid from 300 flowers and the other produced two tetraploids from 400 flowers. This represents frequencies of 3.33 and 5.00 $2n$ eggs per 1000 flowers, respectively. This high $2n$ egg production might be genetic in origin, or it may reflect differences in greenhouse versus field conditions. As little haploid pollen was present in the pollination cage, the high $2n$ egg frequency may also reflect lack of competition between haploid and diploid pollen for $2n$ eggs.

Discussion

Use of $2n$ gametes can help overcome the inbreeding that is inherent in somatic chromosome doubling (Peloquin 1981). This is especially significant, as increasing heterozygosity underpins heterosis in autopolyploids (Mendiburu et al. 1974; Bingham 1979). Results of the crosses reported in this research would suggest that BSP can be a useful source of tetraploids for red clover breeding in the future. Identification or development of additional genotypes that produce $2n$ eggs should not be a limitation, as $2n$ eggs have been found in several different stocks.

Considering the results of the second experiment, prior identification of $2n$ egg producing plants may not be necessary. The use of a wide range of male sterile females (perhaps incorporating several different male sterility genes) could allow the rapid development of a broad based tetraploid population through BSP. If only one male sterile allele is used, 1/36, or 2.78% of the plants in subsequent generations, would be male sterile. This shortage of pollen should not affect seed set too greatly.

In addition, den Nijs was able to increase the frequency of $2n$ egg production in potatoes 500- to 1000-fold through selection, indicating that the trait is under genetic control (den Nijs 1977). In the red clover population reported here, frequency of $2n$ pollen producers is lower than that of $2n$ egg producers, but also under genetic control. This should enable breeding for a population which produces $2n$ pollen at a high frequency. Such a population is currently under development.

It is evident from these results that, at least in red clover, a triploid intermediary is not necessary for the evolution of tetraploidy. Not only were more tetraploids produced than triploids, but red clover triploids are usually sterile (Taylor and Giri 1983). Although the triploids observed in this study appear fertile, their ability to produce tetraploids has not been completely ascertained at this time.

The question remains as to why tetraploid clover has not arisen previously from BSP. One possibility might be that a higher frequency of $2n$ gamete production must exist in a population before BSP is likely to occur. Frequencies of $2n$ gametes in $2x$ potatoes (Camadro and Peloquin 1980; Leue and Peloquin 1980) and in $2x$ alfalfa (Bingham and McCoy 1979) appear to be higher than the frequencies of $2n$ eggs reported here, or of $2n$ pollen reported previously (Parrott and Smith 1984). This may be why BSP occurred in only one of four crosses where $2n$ eggs and $2n$ pollen were present in the first experiment. Even if a tetraploid plant were to arise by BSP, unless the original population produced enough $2n$ gametes for USP to occur, the new tetraploid may be reproductively isolated. Availability of $2n$ gametes for USP is likely to vary from year to year because frequency of $2n$ gamete production is sensitive to environment. This is evidenced by the fact that 'Arlington'-A produced 0.215 $2n$ eggs per 1000 flowers in 1982, but 0.138 in 1983. Further, while $2n$ eggs were tentatively detected in 'Arlington'-D, C760-B and 'Florex'-F in 1982, none were detected in 1983.

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