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# The genetic characterisation of novel multi-addition doubled haploid lines derived from triticale × wheat hybrids

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**Abstract.** Two novel 46-chromosome doubled haploid lines, W66 and M17, derived from separate hexaploid triticale × bread wheat crosses, were characterised using cytological and biochemical markers. Both lines were shown to be relatively stable cytologically, over 11 and 8 generations of selfing, respectively. By examining mitotic and meiotic chromosomes, the stabilities of the two lines were shown to be similar with frequencies of 2n = 46 in 74.2–85.5% of cells. However, over selfed generations, the rye chromosomes were shown to have lost some of their heterochromatin, which made it difficult to establish their continued presence using cytological techniques, such as C-banding alone. Cytological evidence from pairing studies, C-banding, and fluorescence in-situ hybridization, showed that both M17 and W66 are wheat/rye multi-addition lines with rye chromosome constitutions of 1R + 6R, and 1R + 4R, respectively. These conclusions were confirmed by isozyme and storage-protein analysis.

**Key words:** Triticale × wheat – Doubled haploids – Biochemical markers – In situ hybridization

## Introduction

In the Triticeae, the transfer of genes between species has been developed as an important method for crop improvement. Rye (*Secale cereale* L.), in particular, has been shown to be one of the most successful donors for this purpose to wheat since it carries many useful genes to further wheat breeding. The synthesis of triticale is, usually, the first step for introducing rye chromosomes, and this is followed, conventionally, by crossing the derived triticale with wheat. However, this procedure presents problems since in the $F_1$ hybrid no pairing occurs between the R- and D-genome chromosomes resulting, on selfing or backcrossing, in low seed set and sterility in the few progeny that can be produced. An alternative approach is to use the $F_1$ hybrids in anther-culture procedures, and employing this method a number of lines with different rye constitutions have been obtained (Hu et al. 1988). Among a population of such lines, some were found with the unusual chromosome constitution of 2n = 46; these are difficult or impossible to obtain by conventional methods. The genetic characterisation of these new types of wheat, and their stabilities, is the first step in their possible use both for further genetic analysis and for their utility in wheat breeding.

A number of techniques can be used for alien-wheat chromosome identification. Among these, chromosome-banding methods, such as C-banding and N-banding, have been the most widely used. In triticale, for example, rye chromosomes are usually identified by their characteristic heterochromatic bands. However, rye chromosomes can lose their heterochromatin in a wheat background, and to determine the continued presence of rye chromosomes becomes difficult by banding techniques after many selfed generations. Consequently, it becomes necessary to resort to biochemical and molecular markers which have now been shown to be effective for alien-wheat chromosome identification (Gale and Sharp 1988) and usefully complement the cytological techniques of C-banding and in-situ hybridization.

The purpose of the present study was to characterise two novel doubled haploid lines with a chromosome

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constitution of 2n = 46 by analysing cytological and biochemical markers; and to study the stabilities of these two unusual lines by observing both mitotic and meiotic chromosomes.

## Materials and methods

*Materials*

W66 was derived from the cross of triticale cv 'Rosner' × wheat cv 'Kedong 58'; and M17 was derived from the cross of triticale cv 'Beagle' × 'Kedong 58'. Seeds for this study were of the $H_7$–$H_{10}$ generations for W66, and the $H_5$–$H_7$ generations for M17, after selfing from the original doubled haploid plants.

As controls for the analysis of biochemical markers 'Chinese Spring' (CS), its aneuploid lines, and addition lines of chromosomes of rye cv 'Imperial' into CS were also used.

*Cytological analysis*

The number of chromosomes and the chromosomal stabilities of W66 and M17 over generations was determined by examining mitosis in root-tip cells and meiosis in pollen mother cells using the acetocarmine staining procedure as described by Endo (1986). Genomic in-situ hybridization (GISH) using total rye DNA as a probe was used to identify the number of rye chromosomes present in root-tip mitotic cells of M17 and W66, following the technique of Schwarzacher et al. (1992). The presence of particular rye chromosomes was determined by the C-banding technique described by Tao and Hu (1989), and individual rye chromosomes identified according to the standard pattern described by Sybenga (1983).

*Analysis of biochemical markers*

Five biochemical marker systems were used to characterize the lines for the different homoeologous groups. However, since the original triticale parents, 'Rosner' and 'Beagle', are known not to possess 2R (Tao and Hu 1989), this homoeologous group was not studied.

*High-molecular-weight (HMW) subunit composition.* Ten-percent polyaceylamide-gel electrophoresis (PAGE) with sodium dodecyl sulphate (SDS) was used for analysis of HMW subunit composition according to the techniques of Payne and Corfield (1979).

*Esterase isozyme profiles.* Isoelectric focusing (IEF) was used to identify both esterase-1 and esterase-5 phenotypes following the method described by Ainsworth et al. (1984) with modifications by Liu and Gale (1990) and Liu (1991).

*Aminopeptidase isozyme profiles.* Isozyme variation for aminopeptidase was examined following the method described by Koebner and Martin (1989).

*Iodine binding-factor profiles.* Iodine binding-factor variation (*Ibf-1*) was analyzed as described by Liu and Gale (1989).

*α-amylase isozyme profiles.* Isozyme variation for α-amylase was evaluated as described by Ainsworth et al. (1987).

## Results and discussion

*The genetic constitution of W66 and M17*

An examination of mitotic cells in acetocarmine-stained root-tip preparations confirmed that both W66 and M17 have a somatic chromosome number of 2n = 46 (data not shown). Meiotic analysis of the lines indicated that pairing was generally regular with 23 bivalents (Fig. 1a, b). C-banding of root-tip preparations showed that W66 clearly carries at least one pair of rye chromosomes, 1R (Fig. 2a), and that M17 carries at least two pairs, 1R and 6R (Fig. 2b). In W66, in particular, a loss of the characteristic heterochromatin bands on the rye chromosomes was observed over selfing generations, and this made it difficult to verify the continued presence or identity of other rye chromosomes. Therefore, GISH was used to verify the presence and number of rye chromosomes in both lines. At the $H_8$ and $H_{10}$ generations, in M17 and W66, respectively, there were clearly four complete rye chromosomes amongst a total chromosome complement of 46 (Fig. 3a for M17). Occasionally, in some root-tip cells, rye telochromosomes and dicentrics were also found (data not shown). Also occasional cells contained only three (Fig. 3b) and sometimes two rye chromosomes, or the rye chromosomes had deletions, indicative of some instability in both lines. Nevertheless, in general, these cytological results indicate that both lines are stable rye double-addition lines.

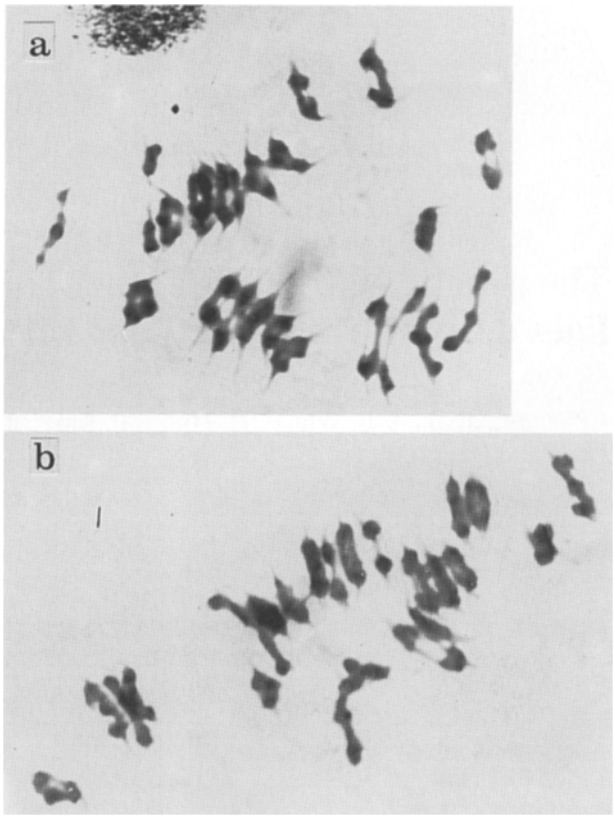


**Fig. 1a, b.** Metaphase-I of meiosis in W66 (**a**) M17 (**b**), showing the presence of 23 bivalents in each line

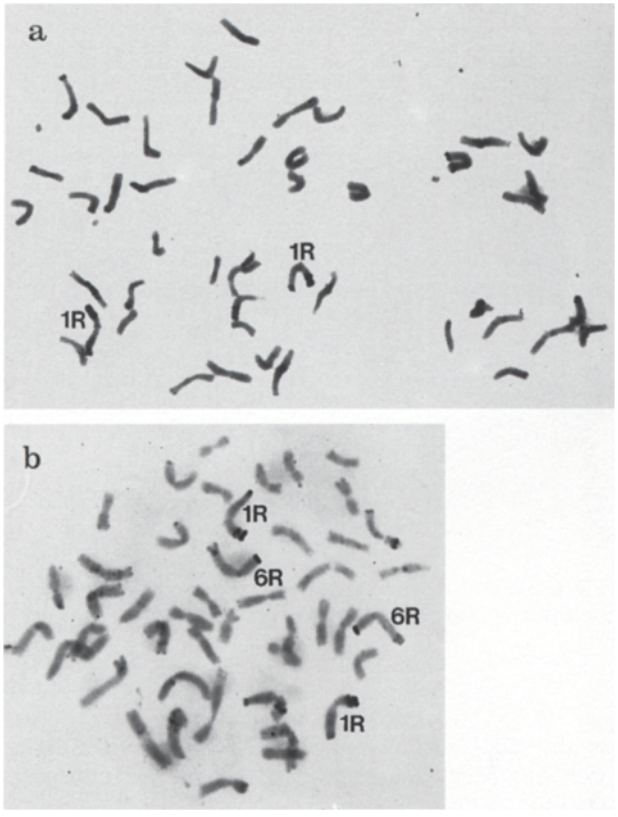


**Fig. 2a, b.** C-banded mitotic metaphase chromosomes of W66 (2n = 46) (**a**) showing the presence of 1R and M17 (**b**) showing 1R and 6R

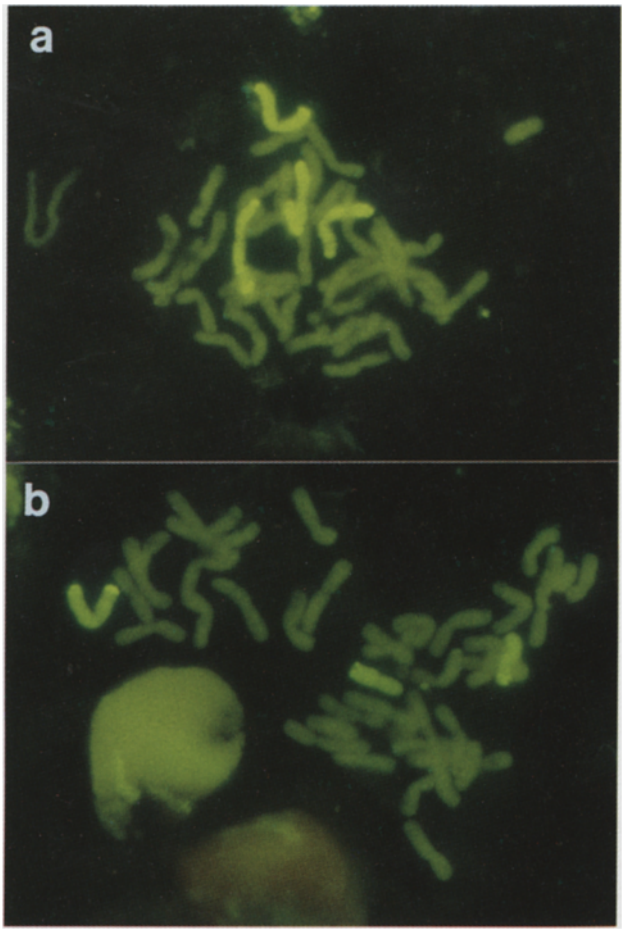


**Fig. 3a, b.** GISH using total rye genomic DNA as a probe. **a** A root-tip cell of M17 showing four rye chromosomes; **b** A root-tip cell of W66 showing only three rye chormosomes, the two on the right of the photograph also appearing to have terminal deletions in one arm

Biochemical markers known to be located on each rye chromosome were then used for further characterization to confirm the identity of the rye chromosomes.

*Homoeologous group 1.* The genes encoding HMW subunits of glutenin, *Glu-1*, are located on the long arms of wheat chromosomes 1A, 1B and 1D and rye chromosome 1R. Thus SDS-PAGE can be used to confirm the presence of the 1R long arms (Lawrence and Shepherd 1981; Payne 1987). As shown in Fig. 4, the SDS-PAGE patterns of reduced glutenins of W66 and M17 showed that rye chromosome 1R is present in both lines, which agreed with the cytological results. However, both M17 and W66 also contained additional bands not present in either parental controls. This may indicate that the original parents which formed the $F_1$ were slightly different genetically from the controls used here, perhaps due to heterogeneity in the original lines.

*Homoeologous group 4.* Genes encoding aminopeptidase-2 are reported to be located on wheat chromosomes 4A, 4B and 4D and rye chromosome 4R (Koebner and Martin 1989). A comparison of the parents 'Rosner' and 'Kedong 58', with W66 showed that rye chromosome 4R is present in W66 (Fig. 5), but absent in M17 (data not shown).

*Homoeologous group 6.* Through analysis of *Est-5* variation, controlled by genes located on rye chromosome 6R (Artemova 1982), it was shown that 6R is absent from W66. However, as predicted from the cytological studies, the presence of 6R in M17 was confirmed (Fig. 6).

*Homoeologous groups 3, 5 and 7.* Three other biochemical marker systems, *Est-1* (for 3R), *Ibf-1* (for 5R), and α-amylase (for 7R), were also used to characterize W66 and M17 (Hart and Langston 1977). No evidence for the presence of either 3R, 5R or 7R in the two doubled haploid lines was obtained (data not shown).

All of the above results lead to the conclusion that W66 is a double-disomic addition line with a rye chromosome constitution of 1R and 4R, and that M17

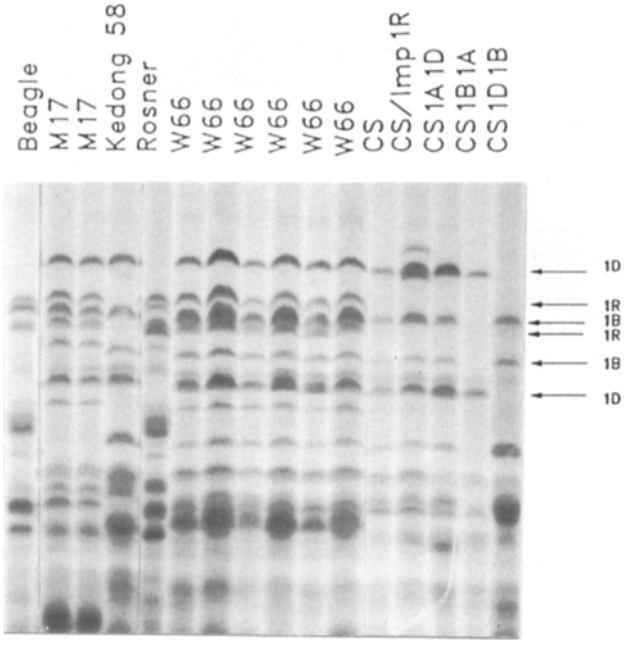


**Fig. 4.** SDS-PAGE patterns of individual plants of W66 and M17, parents, and controls. (*CS1A1D* = Chinese Spring nullisomic 1A-tetrasomic 1D; *CS1B1A* = nullisomic 1B-tetrasomic 1A; *CS1D1B* = nullisomic 1D-tetrasomic 1B), showing the presence of 1R HMW subunits

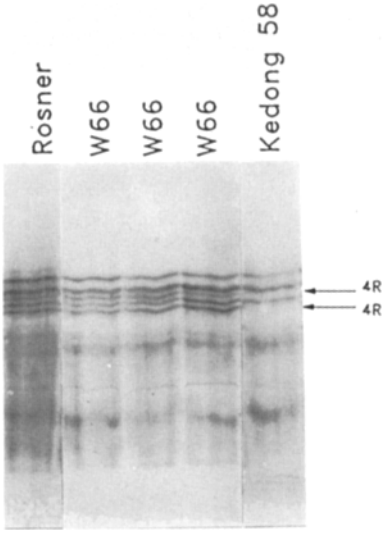


**Fig. 5.** Aminopeptidase-2 IEF phenotypes of individual plants of W66 and its parents showing the presence of 4R in W66

is a double-disomic addition line with a rye chromosome constitution of 1R and 6R.

*The chromosomal stabilities of W66 and M17*

Chromosomal variation has frequently been reported in regenerants derived from anther culture (De Buyser et al. 1985; Charmet et al. 1986). However, little attention has been focused directly on the chromosomal stability of lines in somatic cells or over selfing generations. A study of the stability of these unusual multi-addition lines was, therefore, carried out. From 1980 to 1991 seed of 11 generations of W66, and eight of M17, were accumulated, and the later generations studied for chromosome constitution by examining mitotic and meitotic cells in acetocarmine-stained preparations.

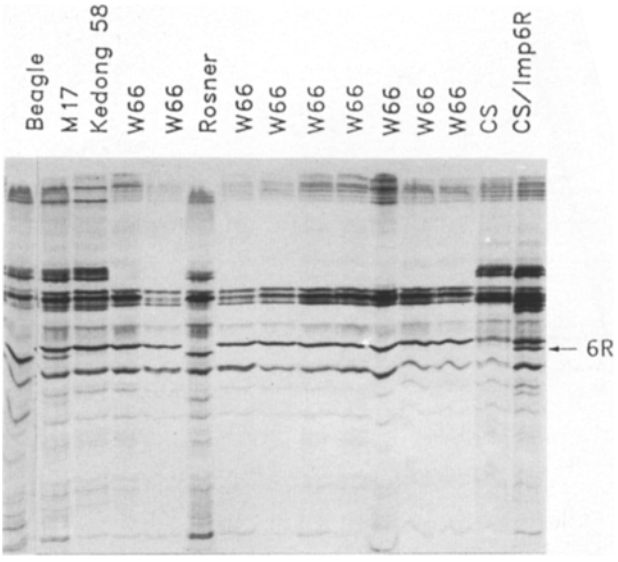


**Fig. 6.** Esterase-5 patterns of individual plants of W66 and M17, parents and controls, showing the presence of 6R in M17

Somatic stability was measured as the frequency of root-tip cells which retained the 46-chromosome constitution. From studies of several plants within each generation the cytological data showed that W66 and M17 had similar somatic stabilities, where the stability of W66 was 78.3–83.3% in mitotic cells from the 7th to 10th generations (Table 1), and the stability of M17 was 74.2–85.5% from the 5th to 7th generations (Table 2). Meiotic stability was measured as the expected frequency of pollen grains with 23 chromosomes, estimated from chromosome pairing studies of PMCs. In W66, 75.5–84.7% of pollen grains were expected to be of n = 23 in plants from the 8th to 10th generations (Table 1), with 76.0–84.4% in M17 plants from the 5th to 7th generations (Table 2). These results suggest that there is little difference in the stabilities of W66 and M17 within or over different generations, either in mitotic or meiotic cells. It can be concluded that these multi-addition lines of wheat derived by anther culture, which are difficult to obtain by conventional programmes, can be stably maintained.

In rye/wheat introgressions the loss of the characteristic heterochromatin bands of the rye chromosomes

**Table 1.** Frequencies of different chromosome constitutions in mitotic and meiotic cells of W66

Mitotic cells (root tips)

| Generation | 2n = 42 | 43 | 44 | 45 | 46 | 47 | 48 | % of cells, 2n = 46 |
|---|---|---|---|---|---|---|---|---|
| W66-2-4-10-7-3 ($H_7$) | 2 | 1 | 4 | 3 | 36 | 0 | 0 | 78.3 |
| W66-2-4-10-7-3-10 ($H_8$) | 1 | 1 | 2 | 1 | 25 | 0 | 0 | 83.3 |
| W66-2-4-10-7-3-10-4 ($H_9$) | 3 | 2 | 12 | 3 | 93 | 3 | 1 | 79.5 |
| W66-2-4-10-7-3-10-4-7 ($H_{10}$) | 2 | 1 | 5 | 8 | 115 | 7 | 4 | 81.0 |

Meiotic cells (pollen mother cells)

| Generation | Chromosome configurations | | | | | | | Expected % of pollen grains n = 23 |
|---|---|---|---|---|---|---|---|---|
| | 23II | 46I | 22II + 2I | 45I | 22II + 1I | 22II + t | 22II | |
| W66-2-4-10-7-3-10 ($H_8$) | 21 | 3 | 2 | 8 | 3 | 2 | 3 | 79.4 |
| W66-2-4-10-7-3-10-4 ($H_9$) | 17 | 6 | 3 | 9 | 2 | 1 | 4 | 75.5 |
| W66-2-4-10-7-3-10-4-7 ($H_{10}$) | 32 | 4 | 7 | 6 | 8 | 0 | 4 | 84.7 |

**Table 2.** Frequencies of different chromosome constitutions in mitotic and meiotic cells of M17

Mitotic cells (root tips)

| Generation | 2n = 42 | 43 | 44 | 45 | 46 | 47 | % of cells, 2n = 46 |
|---|---|---|---|---|---|---|---|
| M17-1-7-3 ($H_5$) | 1 | 0 | 3 | 4 | 47 | 0 | 85.5 |
| M17-1-7-3-4 ($H_6$) | 7 | 3 | 5 | 8 | 72 | 2 | 74.2 |
| M17-1-7-3-4-1 ($H_7$) | 5 | 1 | 3 | 6 | 52 | 0 | 77.6 |

Meiotic cells (pollen mother cells)

| Generation | Chromosome configurations | | | | | | | | Expected % of pollen grains n = 23 |
|---|---|---|---|---|---|---|---|---|---|
| | 23II | 46I | 22II + 2I | 45I | 22II + 1I | 22II + t | 22II | 19II | |
| M17-1-7-3- ($H_5$) | 18 | 11 | 3 | 6 | 3 | 2 | 4 | 1 | 76.0 |
| M17-1-7-3-4 ($H_6$) | 27 | 27 | 4 | 10 | 4 | 0 | 5 | 0 | 84.4 |
| M17-1-7-3-4-1 ($H_7$) | 22 | 19 | 2 | 4 | 7 | 1 | 8 | 0 | 77.0 |

over selfing generations to produce modified chromosomes (Gustafson 1982) makes it difficult to identify their continued presence cytologically. In W66, at the $H_{11}$ generation only the 1R chromosome could be observed from C-banding, whereas the other pair of rye chromosomes could not be identified by this technique, although its presence was shown by GISH. M17 had complete 1R and 6R rye chromosomes at the fourth generation, but subsequently the loss of heterochromatin occured where the C-bands disappeared completely on 6R and partially on 1R by the seventh generation.

The loss of the rye heterochromatin may result in greater cytological stability of the multi-addition condition. Previous reports have found that chiasma frequency was increased by one chiasma per pollen mother cell when a modified 7RL (Merker 1976) or a modified 6RS (Roupakias and Kaltsikes 1977) was present in the disomic condition in triticale. Also a previous analysis of early endosperm development showed that triticale lines in which chromosomes 4R and 6R lacked their heterochromatic blocks had a significantly lower frequency of aberrant endosperm nuclei and improved meiotic stability and test weight at maturity than did control lines where blocks were present (Bennett and Gustafson 1982; Gustafson and Bennett 1982). A modified 1R also affected chromosome pairing in a wheat background, although it did not have an effect in rye itself (Naranjo and Lacadena 1980). These observations might explain why the loss of heterochromatin occurred in W66 and M17, and also why these two lines with a complicated rye chromosome constitution could be stably maintained over 11 (W66) and eight generations (M17) of selfing.

The two lines examined here appear as stable, cytologically, as disomic rye/wheat addition lines although not as stable as wheat itself. For example, the 5R disomic addition line has a reported stability of 60–64%, the 4R addition line a stability of 70–74%, and the other five addition lines similar stabilities of 80–95% (Kaltsikes et al. 1984). The composition of W66 and M17 is more complicated than single disomic rye/wheat addition lines, and they may be expected to be less stable. However, as pointed out above, the difference in stabilities among different generations of these two lines was only between 74.2–85.5%, which is similar to that observed in normal disomic addition lines. Nevertheless, it is perhaps suprising that these two

unusual doubled haploid lines are relatively stable, compared to normal alien addition lines.

Stable wheat lines with a chromosome number of $2n = 46$ are difficult to obtain naturally or by conventional crossing since, generally, the probability of fusion between female and male gametes with the same chromosome composition is low because of their rare frequency (Muntzing 1979). However, this is easily achieved by anther culture (Tao and Hu 1989). Using the same crosses as the present study, Tao and Hu (1989) found that gametes with 23 and 24 chromosomes formed a predominant portion of the pollen plants regenerated in their experiments. Thus, anther culture is a valuable technique for producing novel chromosome combinations, which not only have intrinsic interest but can serve as starting points for alien introgressions or as breeding material in their own right.

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