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Cytogenetics and immature embryo culture at Embrapa Trigo breeding program: transfer of disease resistance from related species by artificial resynthesis of hexaploid wheat (*Triticum aestivum* L. em. Thell)

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Abstract

Worldwide wheat (*Triticum aestivum* L. em. Thell, 2n = 6X = 42, AABBDD) breeding programs aim to reorganize genotypes to achieve better yields, environmental adaptation and food quality. The necessary interdisciplinarity for breeding purposes requires an accurate choice of the most appropriate cellular and/or molecular strategies available to be integrated with agronomic approaches in order to overcome the genetic limitation of each cultivated species, at each agroecosystem. Cytogenetics has given a great contribution to wheat genetic studies and breeding, due to viability of chromosomal variants because of homoeology among genomes in this allohexaploid species and the genus *Triticum*. The level of development of cytogenetic techniques achieved over the last 60 years has set wheat apart from other cereal crops in terms of possibilities to introduce genetic material from other species. Cytogenetic approaches have been extensively used in chromosomal mapping and/or resistance gene transference from tribe *Triticeae*-related species. Monosomic analysis, entire chromosomes engineered through single additions and/or substitutions, reciprocal translocation through radiation or manipulation of homoeologous pairing, as well as synthesis of new amphiploids to allow homologous recombination by chiasmata evolved considerably since the past decades. The association of tissue culture and molecular biology techniques provides bread wheat breeding programs with a powerful set of biotechnological tools. However, knowledge on genetic system components, cytotaxonomical relationships, cytogenetic structure and evolutionary history of wheat species cannot be neglected. This information indicates the appropriate strategy to avoid isolation mechanisms in interspecific or intergeneric crosses, according to the genome constitution of the species the desired gene is to be transferred from. The development of amphiploids as "bridge" species is one of the available procedures to facilitate gene flow between wheat and related species. Since the environment at the center of origin of wheat in Southern Asia is quite different from subtropical environments, Brazilian breeding programs overcome more challenges to adapt wheat crop to biotic and abiotic stresses than some other countries. The germplasm bank of Embrapa Trigo has about 1000 registered entries of *Triticum* relatives, *Aegilops*, *Secale* and *Agropyron* species supplied from several germplasm banks distributed over the world which were multiplied and/or selected for naturally occurring or artificially inoculated fungal diseases. Since *Aegilops squarrosa* L. entries showed very good performance, the genetic variability observed in this species was firstly exploited. It is reported here the strategy used for transferring useful genes from *Ae. squarrosa* (DD, 2n = 14): crossing with tetraploid species (AABB, 2n = 28), rescue and *in vitro* culture of immature embryos for regeneration of the trihaploid (ABD, 2n = 21) hybrid, and colchicine treatment for genome duplication resulting in the artificial synthesis of hexaploid wheat lines (AABBDD, 2n = 42). Results of 10,739 artificial pollinations involving 28 cross combinations amongst eight *T. durum* L., *T. dicoccum* and *T. cartlicum* tetraploid entries used as female parents and ten selected *Ae. squarrosa* sources of resistance as male parents are presented here. Immature embryos from 18 cross combinations were recovered and cultured *in vitro*. Green plantlets from 13 combinations were regenerated. Fertile amphiploids were recovered only from crosses among entries of tetraploid *T. durum* and diploid *Ae. squarrosa.* They originated 11 fertile synthetic amphiploid lines from seven different combinations. Useful stem and leaf rust as well as powdery mildew resistance for future use in breeding programs were obtained.

INTRODUCTION

The importance of wheat (*Triticum aestivum* L. em. Thell 2n = 6x = 42, AABBDD) as food crop comes primarily from the viscoelastic properties of its endosperm gluten proteins for bread production, representing the food basis for western civilization development. The tribe *Triticeae* contains three of the major cereals - barley, rye and wheat - being used by humankind since prehistoric times, as well as the first and unique man made cereal *Triticale*. Octoploid (2n = 8x = 56, AABBDDRR) and hexaploid (2n = 6x = 42, AABBRR) triticale are obtained crossing hexaploid wheat and rye (2n = 6x = 42, AABBDD x 2n = 2x = 14, RR) or tetraploid wheat and rye (2n = 4x = 28, AABB x 2n = 2x = 14, RR) (Gregory, 1987). Several other members of this tribe are also important as forage and pasture grasses (Miller, 1987).

Wheat domestication occurred in primitive farms of Southwestern Asia, at the "Fertile Crescent" of Mesopotamia, between 7.000 and 9.000 BC (Bell, 1987). Since

the beginning of plant breeding about 200 years ago, quite impressive improvements in yield, bread making quality, plant architecture, and increased resistance to biotic and abiotic stresses were obtained. The total number of accessions in national and local gene banks around the world has been estimated in about 400.000 entries although there may be duplications (Poehlman and Sleper, 1996).

Worldwide wheat breeding programs aim to reorganize genotypes to achieve better yields, adaptation to different agroecosystems and food quality. Genetics is the central discipline of plant breeding, but not the unique one. The desired expression of the great majority of agronomic characteristics in cultivated plants depends on better genotypes as well as an appropriate management with the environment.

The progressive integration of classical and modern methodologies derived from several biological disciplines into plant breeding programs broadens the possibility of reorganizing the genomes of cultivated species.

From a pragmatical point of view, for crop improvement programs to supply the useful demand for useful genetic products it is necessary a deep understanding of the role of meiotic and breeding systems, which are the two main components of each cultivated species genetic system that define the nature, the extent and the release of genetic variability in plants (Lewis and John, 1965).

Therefore, the necessary interdisciplinarity to better achieve breeding purposes requires an accurate choice of available and more appropriate cellular and/or molecular strategies. It is also necessary to integrate them with agronomic approaches in order to overcome the genetic limitations for the cultivation of distinct species at different agroecosystems (MacKey, 1970).

Since the environmental conditions prevailing at the center of origin of wheat are quite different from subtropical agroecosystems, Brazilian breeding programs face additional challenges to adapt wheat crop as compared to some other countries. Several biotic and abiotic stresses are environmentally circumvented by soil correction, different planting dates, crop rotation systems, as well as biological and/or chemical control of pathogens.

Nevertheless, when plant breeders obtain a distinct level of genetic adaptation, the use of cultural practices and/or agrochemicals may be reduced, increasing the protection of the environment, animals and human health. Better yields may also mean, as pointed out by Dr. J.M. Valls, avoidance of agricultural utilization of unexploited natural ecosystems, leading to desired environmental preservation.

Related species are an important pool of genes for resistance to biotic and abiotic stresses (Sears, 1965; Riley and Kimber, 1966; Knott and Dvorak, 1976). However, the exploration of such useful genes in crop breeding is limited by several isolation mechanisms between species usually observed in interspecific or intergeneric crosses (Feldmann, 1976, 1977; MacKey, 1987).

Such genetic barriers may result in: 1) no fertilization at all, 2) hybrid inviability occurring at different stages of plant development, or 3) hybrid sterility. When hybrid sterility is caused only by lack of homologous chromosomes, new amphiploids may be easily obtained through genome duplication with colchicine.

Consequently, the knowledge of cytotaxonomical relationships, cytogenetic structure and evolutionary history of the species involved in the crosses indicate the most appropriate procedure necessary to avoid isolation mechanisms according to the genome constitution of the species presenting the gene to be transferred (Knott, 1989; Darlington, 1973).

At the same time, hybridization between species depends on several experimental and environmental variables that may be circumvented by crossing different genotypes, changing male and female parents, using other species as bridges, *in vitro* culturing immature embryos and cytologically selecting euploid and resistant plants in segregant progenies (Riley and Kimber, 1966).

Cytogenetics, as a discipline that deals with the mechanical basis of Mendelian inheritance, is of fundamental importance to wheat breeding programs. The great contribution of chromosomal engineering to the understanding of wheat genetics is due to the viability of aneuploid chromosomal variants and the discovery of the role of polyploidy in the evolution of *Triticeae*.

The determination of the basic chromosome number (x = 7) in diploid, tetraploid and hexaploid wheats by Sakamura (1918) and extensive genome analysis by Kihara (1919, 1924), as reviewed by Lilienfeld (1951), was followed by the establishment of homoeology between genomes by Sears and Okamoto (1958) and Riley *et al*. (1958).

The use of colchicine for artificial duplication of genomes (Blakeslee, 1937) and the development of artificial media for embryo rescue and *in vitro* culture by Jenkins and Mochizuki (1957) were essential for the artificial increment of gene flow by introgression in the tribe *Triticeae*. The degeneration of hybrid endosperm would be overcome by embryo rescue and *in vitro* regeneration of hybrid plants, while sterility would be circumvented by treatment with colchicine for genome duplication.

In the past decades, classical cytogenetics and tissue culture techniques provided bread wheat breeding programs with a powerful set of biotechnological tools which nowadays are being complemented by molecular biology techniques.

The simultaneous use of *in vitro* culture of immature embryos, followed by cytological selection of plants with balanced euploid chromosome number and agronomic characteristics to be introduced in interespecific or intergeneric progenies, constitute the basis for transferring innumerous new genes of disease resistance (Sears, 1956, 1972; Riley and Kimber, 1966; Sharma and Knott, 1966; Cauderon, 1981; Gale and Miller, 1987; Worland *et al*., 1987).

Cytogenetic information is also necessary when using haplodiploidization after intervarietal crosses for breeding purposes or for genetic studies, either via androgenesis (through *in vitro* anther or microspore culture) or via gymnogenesis (through *in vitro* immature embryos culture), to achieve complete homozigosity in only one generation (Moraes-Fernandes *et al*., 1991, 1999; Picard *et al*., 1994; Peters *et al*., 1999).

The impressive advances on cellular and molecular methodologies that are coming into widespread use in breeding programs are progressing more slowly in bread wheat, as occurred in the past with other genetic approaches, because of the peculiarities of the genetic system of *T. aestivum* (Day and Lupton, 1987).

The availability of newer molecular marker systems such as AFLP (amplified fragment length polymorphism) and microsatellites may increase precision and save time to achieve better levels of resistance to either biotic or abiotic stresses in wheat. AFLP and microsatellites are both PCR based and amenable to automation, permitting the simultaneous screening of dozen of loci and detection of higher levels of polymorphism than other systems (Fedak, 1998).

On celular biology, the microspore technique for androgenetic haplodiploidization, already available for canola and barley, is progressing in wheat (Kasha *et al*., 1998).

The research on plant transformation for disease resistance, primarily using antifungal proteins that act against the pathogens is still in inicial stages but presenting very promising preliminary results (Bushnell *et al*., 1998; Barcelo *et al*., 1998; Blechl and Anderson, 1998; Chibbar *et al*., 1998; Fry *et al*., 1998; Higgins *et al*., 1998).

As stated by Snape (1987), stresses is an area of genetic ignorance that in the future will be confidentially much reduced. Aneuploid methods and emerging molecular techniques will facilitate and add precision to genetic analysis of useful genes.

BREAD WHEAT GENETIC SYSTEM

Triticum aestivum L. em. Thell (2n = 6x = 42) is an autogamous alohexaploid species (AABBDD) that combines the genomes of three diploid ancestrals (Peterson, 1965). The donors of the A and D genomes, *T. monococcum* (2n = 14, AA) and *Aegilops squarrosa* (2n = 14, DD) were already clearly identified by genomic analysis (Morris and Sears, 1967).

The great amount of worldwide studies trying to clarify wheat origin and evolution during this century gave rise to several different taxonomic propositions for the genus *Triticum* and tribe *Triticeae* species nomenclature. According to different authors, many names may be used for the same species, as is the case of *Ae. squarrosa* and *T. tauschii* (Bowden, 1959; MacKey, 1970; Feldmann, 1976).

Experimental crosses between *T. monococcum* (AA) and *Ae. squarrosa* (DD) and bread wheat (AABBDD) resulted in pairing between the chromosomes of the diploid species and the hexaploid wheat at meiosis (7 II,14 I) in both AABD and ABDD hybrids. In the process of becoming an alohexaploid species, six of the seven A chromosomes of wheat genome are still fully homologous to *T. monococcum*, while the seven D chromosomes are fully homologous to *Ae. squarrosa* (Lilienfeld, 1951; Riley, 1965) (Figure 1).

However, the donor of the B genome is still controversial and believed to be extinct, very modified or not yet discovered. Many diploid-related species studied showed partial homology and chromosome pairing so far but not to the extent observed for the A and D genome donors. *Ae. speltoides* (2n = 14, BB?) is the species generally accepted as the probable donor of the B genome (Sarkar and Stebbins, 1956; Dvorak, 1972; Miller, 1987; Yan *et al*., 1998) albeit the conflicting results between genome analysis based on chromosome pairing and molecular data. Variation on restriction fragments of repeated nucleotide sequences (RNS) suggests that the B genome is indeed related to *Ae. speltoides* (Dvorak, 1998).

MAPPING CHROMOSOMES AND GENES IN WHEAT GENOME

The discovery by Sears (1939, 1944) of a spontaneous haploid plant of "Chinese Spring" wheat cultivar was the beginning of the monumental work of chromosomal engineering in this species. Because of the buffering effect of polyploidy, viable descendents presenting wide range of chromosome number were obtained from the artificial pollination of ABD trihaploid plants by AABBDD (2n = 42) hexaploids.

Sears selected 21 phenotypically distinct monosomic plants (2n = 41) that were assigned by Roman numerals from I to XXI and later by numbers and genome designating letters. The monosomics of each chromosome pair were identified by Sears (1954, 1958) based on their phenotype. Since the majority of wheat chromosomes is very similar in size and centromere position, the author distinguished them by phenotypic differences. Distinct banding patterns obtained from more recent studies make possible to identify specific chromosomes through cytological examination (Flavell *et al*., 1987) .

The use of monosomic series on chromosome mapping is based on somatic chromosome countings and cytological selection. In each of the 21 monosomic families (2n - 1), the plants segregate giving rise to 2n, 2n - 1 and 2n - 2 seeds. The 2n - 1 plants need to be cytologically identified before pollination by the cultivar to be mapped and the Mendelian segregation pattern of the character is statistically tested (qui-square) in the 21 F_2 progenies.

In those progenies presenting distorted Mendelian segregation, the missing chromosome is the critical one, where the genetic factor coding for the character is located. This method evolved to chromosomal engineering to con-

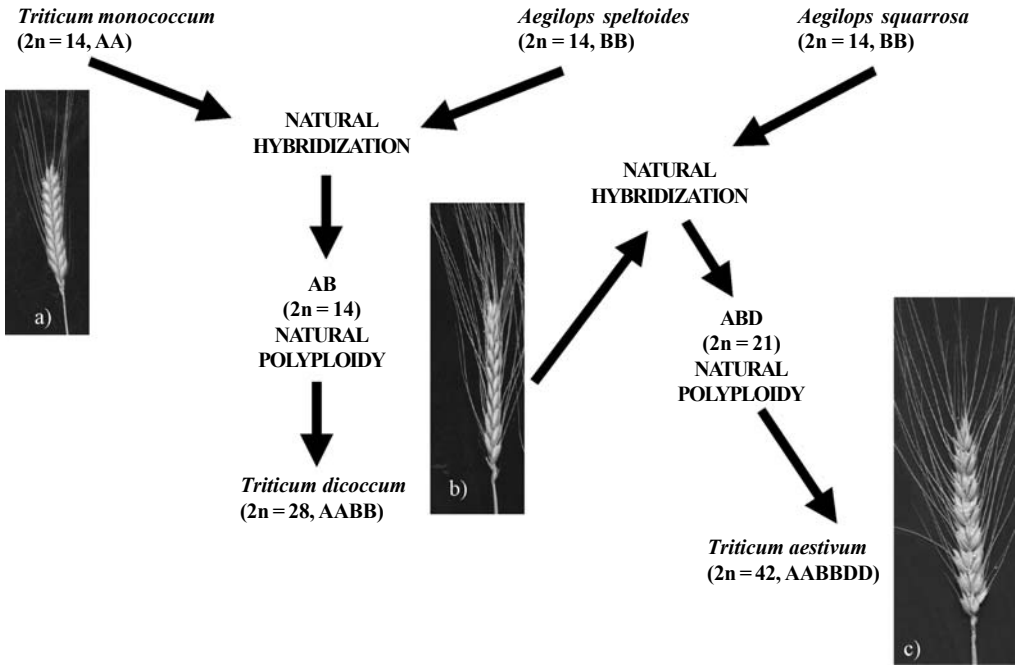

Figure 1 - Synoptic chart of cultivated wheats evolution: the diploid (2n = 14, AA) forms of *Triticum monococcum* (**a**) were naturally pollinated by weed species, possible *Aegilops speltoides* (2n = 14, BB?), in about 10,000 B.C. primitive farms. The subsequent genome duplication of hybrids by natural polyploidy gave rise to several wild and cultivated tetraploid species (2n = 28, AABB) like *Triticum dicoccum* (**b**) and *Triticum durum* (Figure 2a); again, the natural pollination of the tetraploid *T. dicoccum* (**b**) by another weed species, *Aegilops squarrosa* (2n = 14, DD) gave rise to the hexaploid (2n = 42, AABBDD) species (**c**).

struct addition and substitution lines to make genetic analysis of each individual chromosome and background genotypic effects possible (Kimber, 1977; Law *et al*., 1987).

The three wheat genomes (A, B, and D) were found to be partially genetically related. Many genes are duplicated or triplicated meaning four to six alleles at some loci instead of the characteristic two of diploid species (Riley *et al*., 1959).

Aneuploid studies proved the genomic homoeology among the majority of the chromosome pairs of each genome. Plants engineered to be nullisomic for one pair of chromosomes (2n - 2) from one genome but tetrasomic (2n + 2) for the equivalent pair on the other two genomes (nuli/tetrasomic) have the normal phenotype restored (Sears, 1966).

This genetic homoeology among genomes may distort the inheritance patterns of segregation since polysomy leads to more complex genetic studies and breeding methodologies (Sears, 1972). The occurrence of repeated loci suggest that the three wheat genomes possibly originated from a common ancestor.

Gill and Gill (1998) reported the construction of cytologically based physical maps of molecular markers for all 21 chromosomes of wheat in the past several years. Their work was based on a chromosome breakage system producing deletion stocks: from 436 deletion lines, 291 involving all 21 chromosomes were selected to map 384 markers. Ten different libraries of various *Triticeae* species were used to detect 908 loci. Because gene collinearity is conserved among A-, B- and D-genome homoeologous chromosomes, with few exceptions, consensus physical maps for the basic seven chromosome groups could be constructed, revealing that wheat genes are present in clusters interspersed by blocks of repetitive sequences visualized as regions of low gene density.

In spite of the genomic homoeology, usually chromosome pairing in wheat is regular, forming 21 pairs at meiosis. The regular pairing is a basic requirement to fertility, genetic stability and varietal uniformity in self-pollinated crops like bread wheat that has the seed as economic product (Riley and Law, 1965).

Chromosome pairing restrict to homologous chromosomes is guaranteed by a gene factor (*Ph1*) located on the 5B chromosome (Sears and Okamoto, 1958; Riley *et al*. 1958). When the long arm of the 5B chromosome is absent, homoeologous pairing between chromosomes of the three genomes is possible and homoeologous translocations may occur. This gives the possibility of recombining chromosomes of wheat and other members of the tribe *Triticeae*.

Recently, Aragón-Alcaide *et al*. (1996, 1997) have reported the isolation of a DNA sequence (CCS1) specific to the centromere of chromosomes of cereals and other *Triticeae* grasses. The results obtained in the hybridization experiment suggest the existence of a structural difference between the centromeres of *Ph1*-present and *Ph1*-deficient genotypes (Miller *et al*., 1998).

CYTOGENETICS AND EVOLUTION: STRATEGIES FOR GENE TRANSFERENCE FROM RELATED SPECIES TO WHEAT

Several useful cytogenetic strategies were developed to manipulate the chromosomes of part or the whole genome of a species for the improvement of bread wheat.

Many useful genes from wild species are available to be used in breeding programs as source of genes for disease resistance applying different chromosomal engineering techniques involving backcrosses, "bridges" species and selection of cytological as well as desired agronomic characteristics (Knott and Dvorak, 1976; Stalker, 1980; Dhalival *et al*., 1986; Gale and Miller, 1987).

The transfer of alien genes depends on the passage of a piece of chromosome from the donor species to the recipient species, which have to be as small as possible in order to avoid transferring undesirable characteristics. Obviously the transference will be easier if the donor species have chromosomes fully homologous to bread wheat, allowing the occurrence of crossing over and genetic recombination via chiasmata.

Riley and Kimber (1966) reviewed the possibility of transferring genes from related species to bread wheat by evaluating artificial amphiploids and chromosomal addition and substitution lines as well as translocated chromosomal segments. They concluded that, instead of trying to create new species of cereals like *Triticale*, it would be more useful to transfer the minimum alien variation into cultivated crops in order to avoid genetic unbalance in genomes that required so many generations to be adjusted by natural selection. They stated that *Triticale* is the only artificial amphiploid being used in agriculture.

After McFadden and Sears (1944, 1946) resynthesized a hexaploid wheat, the wide development of new amphiploids has been possible if treating the haploid hybrid with colchicine after artificial hybridization for the duplication of the genomes. Gene flow is thus possible using the artificial amphiploid as a "bridge species" because of the obvious better crossing compatibility of hexaploid wheat with species of the same or more similar ploidy levels.

The transference of genes involving small chromosomal segments may occur via chiasmata if there is genome homology between wheat (the receptor genome) and the species the gene(s) is(are) being transferred from (the donator genome). However, irradiation or manipulation of the 5B system to induce chromosomal breaks is indicated when the transference is made from homoeologous or non-homoeologous species through reciprocal translocations. In this case, it is necessary to build up specific chromosomal additions or substitution lines carrying the resistance gene(s) (Riley and Kimber, 1966).

The characteristic triplication of bread wheat genetic material facilitates gene transference between related species since the effects of deleterious genes present on an alien chromosome substitution from one genome is masked by the presence of duplicated genes on the other two genomes.

Besides, the hexaploid plant can tolerate loss or addition of all chromosomes or segments of chromosomes without drastic effects on its viability. These factors, together with the level of development of aneuploid cytogenetic techniques achieved over the last 60 years, have set wheat apart from other cereals in terms of the possibilities for the introduction of genetic material from related species (Gale and Miller, 1987).

Nowadays, transgenesis emerges as a very promising tool for the manipulation of genes more distantly related and also for cloning and transferring genes from other *Triticeae* species. Indeed transgenesis will provide a wider and easier profit for wheat breeding programs in the near future, but cytogenetics knowledge and training will most certainly continue to be necessary.

HOMOLOGOUS AND HOMOEOLOGOUS WHEAT-RELATED SPECIES

It is obviously easier to use the diploids *T. monococcum* (AA), *Aegilops squarrosa* (DD), and the tetraploids *T. turgidum*, *T. durum* and *T. dicoccum* (AABB) to transfer genes into bread wheat because of the homology between their chromosomes and the chromosomes of the cultivated hexaploid wheat.

If a resistance trait is identified in *T. monococcum* (2n = 14, AA), it is mostly indicated to use the tetraploid species (2n = 28, AABB) as a bridge and backcross the triploid hybrids (AAB) to the tetraploid (AAB x AABB) (see Kerber and Dyck, 1973).

Before backcrossing, the euploids with 2n = 28 chromosomes and expressing the characteristic being transferred have to be selected at each generation. Similar procedure is recommended for the transference of genes from the tetraploid to the hexaploid level (AABB x AABBDD).

If the resistance trait is identified in *Aegilops squarrosa* (2n = 14; DD), the transference may be done by crossing *Ae. squarrosa* with a tetraploid species (AABB x DD). The ABD haploid embryos need to be *in vitro* cultured due to *in situ* endosperm degeneration.

The artificial resynthesis of fully homozygous hexaploid wheat carrying the desired gene from the D genome is accomplished by the duplication of the genomes with colchicine. The new synthetic bread wheat may be used directly in different breeding programs without any additional special procedure (Kerber and Dyck, 1969).

Useful genes from other species of the tribe *Triticeae* (*Aegilops*, *Haynaldia*, *Agropyron*, *Secale* and *Hordeum*) that normally do not pair with hexaploid wheat chromosomes are more difficult to transfer into bread wheat. The chromosomal engineering methodology may be very sophisticated depending on the genetic system of each species (Knott, 1961).

The classical models are 1) the transference of rust resistance from *Ae. umbelullata* using radiation to induce translocation that originated the hexaploid wheat line named transfer (Sears, 1956), and 2) the transference of resistance to yellow rust from *Ae. comosa* to a line named compair through the manipulation of the 5B system using a chromosome addition line crossed to *Ae. speltoides* that has the same pairing gene (Riley *et al*., 1968).

Innumerous studies on meiotic pairing of intergeneric hybrids indicate that homoeology is very common between other genus of the tribe *Triticeae* (Cauderon, 1981). Molecular studies indicating collinearity of distantly related species confirm the observations of homoeologous chromosome pairing between *Triticeae* genomes (Gill and Gill, 1998).

LOOKING FOR GENETIC VARIABILITY TO DISEASE RESISTANCE IN WHEAT-RELATED SPECIES AT EMBRAPA TRIGO

The main challenges, regarding stresses, faced by bread wheat breeders in southern Brazil are: 1) to overcome toxic levels of aluminum and manganese in the soil; 2) high variability within the fungi causing stem and leaf rust diseases introducing new races almost every year; 3) severe epidemics of necrotrophic diseases caused by a complex of pathogens, mainly those inducing leaf spots, roots, and head diseases; 4) prolonged rainy and cloudy days causing poor soil drainage and light stresses that may favor the attack of fungi; 5) strong winds during spring, and 6) drastic climatic fluctuation at flowering time causing male sterility, with frequent temperature drops of about 20°C in less than 24 h that may often be followed by severe frosts (Caetano, 1988; Moraes-Fernandes *et al*., 1991).

Some of these events may be controlled by resistance conferred by genes existing in local varieties or by soil management as for aluminum toxicity. However, water logging and light stress cannot be controlled by man.

Depending on the year, wheat losses due to diseases vary from 8 to 40% (Luz, 1984; Picinini *et al*., 1993). Brazilian wheat cultivars are worldwide considered to have a high level of resistance to several diseases. Nevertheless, this resistance may not be enough to overcome severe epidemics that occur in some extremely variable winters in southern Brazil, as well as new races of rust fungus.

Therefore the genetic variability available to be explored by wheat breeding programs may be amplified by new genes originated from wild species (Moraes-Fernandes, 1990).

The first access of wild species were introduced with the assistance and kindness of Dr. Eric Kerber, from the Winnipeg Experimental Station, Canada, who supplied Embrapa Trigo, in Passo Fundo, RS, Brazil, with about 200 samples of his own collection of wild species, mainly *Aegilops squarrosa*, and technical assistance for the project on wide crosses started in 1979 (Moraes-Fernandes *et al*., 1980).

New entries were obtained in 1985 from several regions of the world presenting winter environmental conditions similar to southern Brazil (Moraes-Fernandes *et al*., 1988). The purpose of these introductions was to amplify the possibilities of finding better genes for resistance to diseases occurring in southern Brazil.

Up to now the germplasm bank of Embrapa Trigo has about 1000 registered entries belonging to different species from several genus such as *Agropyron*, *Aegilops*, *Hordeum*, *Secale* and *Triticum*.

Seeds of new introductions were increased locally. However, high levels of pollen and seed sterility were observed in many entries of the species *Triticum monococcum* (AA, 2n = 14) whenever typical southern Brazilian winter drastic temperature oscillations occurred.

On the other hand, entries of *Ae. squarrosa* (2n = 14; DD) developed very well in Passo Fundo and did not show pollen or seed sterility. Besides that, according to Dr. Eric Kerber's personal observation, the size of *Ae. squarrosa* spikes produced here was more than twice the size of heads from the same entries grown in Canada.

T. monococcum develops better in Canada than here, probably due to less environmental variation.

The presumed donor of the B genome, *Ae. speltoides* (2n = 14, BB?), developed very badly under Passo Fundo environmental conditions. Hence seeds had to be multiplied in growth chamber with artificially controlled environment.

These observations indicated that the A and B genomes may be less ecologically adapted to southern Brazil than the D genome. It was already suggested that the addition of the D genome led to an increased adaptation of hexaploid wheat to cooler, more temperate climates of central Asia and Europe or Brazil but much more studies in this line of research should be done.

The monosomic analysis of three worldwide important genes found in Brazilian germplasm performed at Embrapa Trigo showed that the gene for resistance to aluminum toxicity identified in the Brazilian wheat cultivar BH 1146 is located on chromosome 4D (Lagos *et al.*, 1991) and the two genes for leaf rust adult-plant resistance identified in the Brazilian wheat cultivar Toropi (Barcellos *et al*., 2000) are located on chromosomes 1A and 4D (Brammer *et al*., 1998). This is only another example of the innumerous interesting new cytogenetic areas that need to be enriched with more research. Reide and Anderson (1996) identified the chromosome arm carrying the gene for aluminum resistance in BH 1146 wheat.

Intergeneric crosses were made between selected *Agropyron elongatum* (= *Thinopyron ponticum*, 2n = 10x = 70) entries and susceptible local wheat cultivars at Embrapa Trigo since 1988. Rescued immature embryos were cultured *in vitro* and the developed plants were backcrossed to wheat and latter on screened for disease resistance (Angra, 1995).

The haplodiploidization of selected progenies through

somatic elimination of pollen donor genome by maize pollination and colchicine treatment is a routine procedure being used at Embrapa Trigo to transfer non-homologous chromosomal segments into local cultivars (Gouvea *et al*., 1997; Angra *et al*., 1999).

Since *Ae. squarrosa* entries presented very good results regarding disease resistance when evaluated at Embrapa Trigo, it was decided to start exploiting the genetic variability observed in this species (Moraes-Fernandes *et al*., 1980; Prestes *et al*., 1994). It is reported here the strategy used for transferring useful genes from *Ae. squarrosa* L (2n = 14, DD) by crossing it with tetraploid species (2n = 28, AABB) to artificially synthesize hexaploid lines of bread wheat.

MATERIAL AND METHODS

Pure lines of *Ae. squarrosa* L. entries kindly supplied by Dr. Eric Kerber were multiplied and stored at the germplasm bank of Embrapa Trigo. Those lines having winter or unknown growth habit were vernalized for 8 weeks at 3°C and cultivated in greenhouse.

The crosses, presented in Table I, were made by emasculating the female plants when pollen grains in the anthers were immature (Figure 2). In order to increase the probability of successful fertilization, the pollination was done several times before and after the ovary could be seen plumulous, an indication of receptiveness.

The flowers were daily observed to identify developing embryos. Best results were obtained in growth chamber than under greenhouse conditions because temperature and humidity control increases the survival rate of hybrid embryos.

Embryo rescue for *in vitro* culture was made 14 to 18 days after the pollination of the flowers to avoid starting endosperm degeneration. Roots of *in vitro*-regenerated green plantlets were pre-treated for chromosome counting.

After acclimatized in growing chamber, the plants were treated with 0.025% colchicine (Figure 3). The duplicated tillers were fertile while those remaining haploids were sterile. The seeds of the new fully homozygous duplicated genotypes were then multiplied for phytopathological tests.

RESULTS

Results of the crosses between *Ae. squarrosa* (DD) entries selected for resistance to diseases and the tetraploid "bridge" species (AABB) are shown in Table I.

A total of 10.739 artificial pollinations were performed in 28 cross combinations among eight *T. durum* L., *T. dicoccum*, and *T. carthlicum* tetraploid entries, used as female parents (2n = 28, AABB) and ten selected *Ae. squarrosa* sources of resistance, used as male parents (2n = 14; DD).

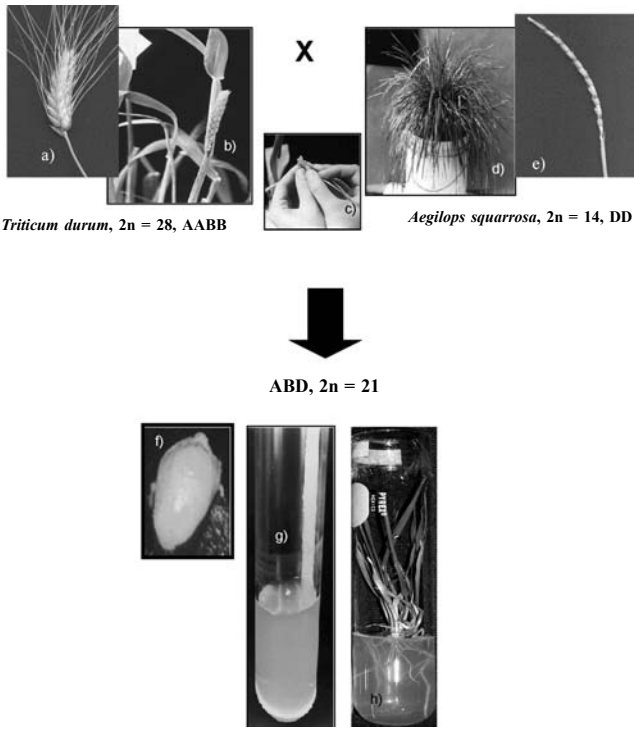

Figure 2 - For the experimental resynthesis of hexaploid wheat, a tetraploid species, *Triticum durum*, 2n = 28, AABB (**a**), is emasculated (**b** and **c**), and pollinated by *Aegilops squarrosa*, 2n = 14, DD (**d**,**e**). The hybrid embryo, 2n = 21, ABD needs to be rescued (**f**,**g**) for green plantlets regeneration (**h**) and genome duplication by colchine treatment (Figure 3).

Immature embryos belonging to 18 cross combinations were rescued and cultured *in vitro* and green plantlets from 13 combinations were regenerated.

The frequency of successful fertilization is indicated by the number of grains developed, from which immature embryos were rescued. The frequency of grains formed varied from zero to a maximum of 19.0%, observed in 1983, in the cross *T. dicoccum* NE 22860/*Ae. squarrosa* NE 29431.

The highest frequency of *in vitro* green plantlets regeneration as well as the number of seeds from fertile plants harvested after colchicine treatment was observed in the cross *T. durum* NE 44665/*Ae. squarrosa* NE 29341. These results demonstrate the importance of trying several distinct genotypes as "bridges". The most resistant entries of *Ae. squarrosa*, NE 20211 and NE 20221, were respectively involved in 8 and 6 different cross combinations before be found one compatible in 1983. Among the 8 tetraploids used only the *T. durum* NE 22912 showed the desired compatibility.

In 1984, crosses using *Ae. squarrosa* as female parent resulted in better levels of successful fertilization and *in vitro* green plantlets regeneration. Nevertheless, no fertility was achieved after colchicine treatment since all plants died before tillering (Table II).

After colchicine treatment, fertile amphiploids were

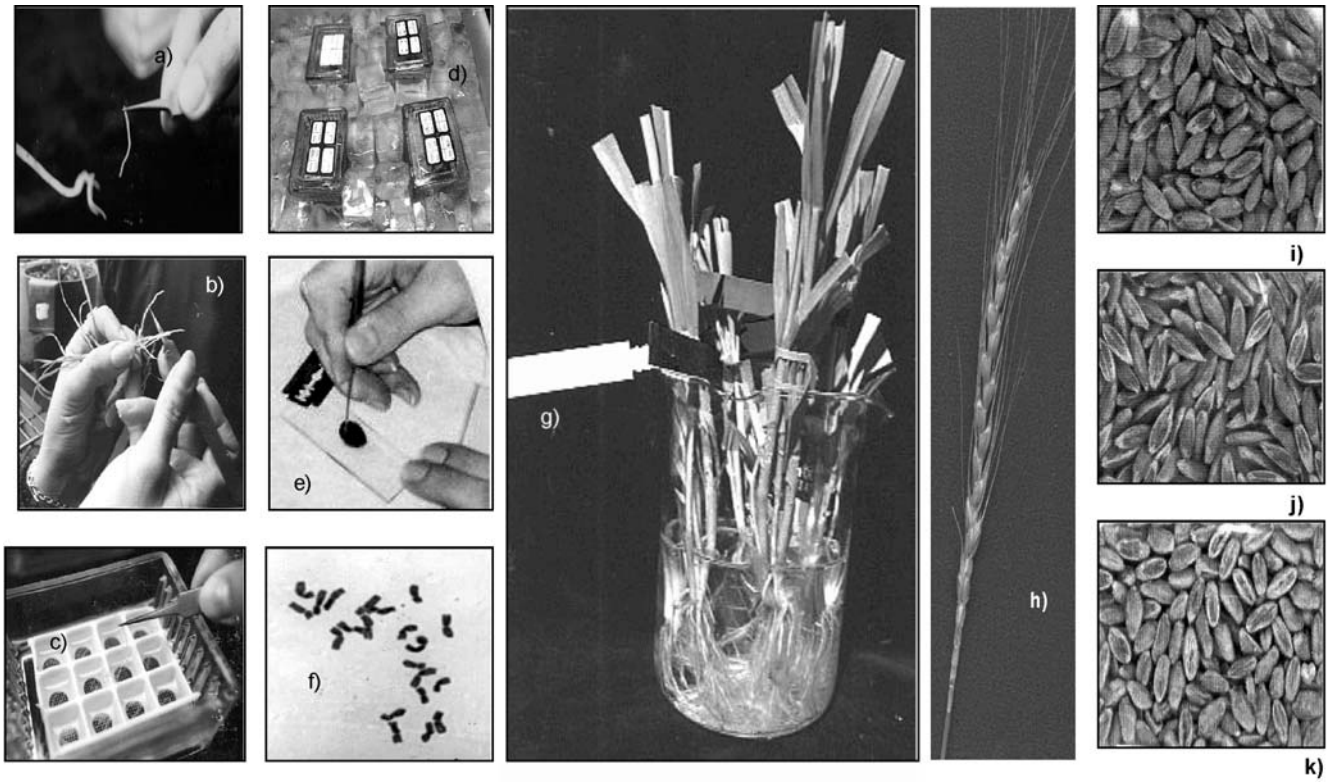

Figure 3 - After root tips ice pre-treatment (**a**,**b**,**c**,**d**), chromosome counting confirms sucessful hybridization (**e**,**f**). The new tillers that will develop after colchicine treatment (**g**), will present genomes duplication and consequently, fertility is restaured (**h**). The new fully homozygous hexaploid lines, 2n = 42, AABBDD obtained (**i**,**j**,**k**), may now be used to transfer the resistance genes either from *T. durum* or *Ae. squarrosa* to susceptible cultivars, by usual crossing procedures of conventional breeding programs without any other special procedure. Seeds of three new synthetic hexaploid lines obtained at Embrapa Trigo (Table I), PF 804001, PF 844004 and PF 844006 are show in i, j and k.

obtained only from the crosses between the tetraploid *T. durum* and the diploid *Ae. squarrosa*, giving rise to 11 fertile synthetic lines from seven different cross combinations.

The Brazilian synthetic hexaploid wheat lines available to breeders since 1985 are presented in Table III.

The results of stem rust reaction of synthetic lines evaluated by E.T. Coelho for the six most important races occurring in southern Brazil in 1986 demonstrate that PF 804001, PF 804002, PF 834001, PF 844002, PF 844004, PF 844005, PF 844007 and PF 844008 showed better levels of resistance to stem rust races *G11*, *G15*, *G18*, *G20*, *G23 and G24* than *Sr33*, the known gene transferred from *Ae. squarrosa*. Since both parents were resistant to these stem rust races, the synthetic hexaploids may have genes from both *T. durum* and *Ae. squarrosa* that can be explored by breeders in their research programs (Moraes-Fernandes *et al*., 1990).

The hexaploid synthetic line PF 834001 was also moderately resistant to powdery mildew, as evaluated by W.I. Linhares. The crosses, embryo culture and chromosome counting to obtain this synthetic hexaploid line were made by S.R. Antoniolli (Moraes-Fernandes *et al*., 1990). The progenitor *Ae. squarrosa* NE 20342 was immune to powdery mildew and *T. durum* NE 22912 was resistant. Hence, the expression of resistance and immunity of both parents was partially inhibited in the new genetic substract, as reported by Harvey *et al*. (1980). Kerber and Green (1980) observed that resistance to rust was suppressed in some synthetic lines.

The synthetics PF 844004 and PF 844005 derived from *Ae. squarrosa* NE 20211 were resistant to all important races of leaf rust, except race B32 and showed variation for race B26, according to the evaluation performed by A.L. Barcellos. The level of resistance to some races was better than that presented by *Lr 21*, which is also derived from *Ae. squarrosa*.

CONCLUDING REMARKS

The alopolyploid nature of wheat and the extensive knowledge of its genome relationships provide plant breeders with a rare opportunity to exploit variation available in alien genera for crop improvement.

Nevertheless experiments on chromosome manipulation to transfer genes between species depend on the evaluation and correct identification of suitable characteristics to be transferred into bread wheat.

Table I - Results of crosses between tetraploid species (AABB) and *Aegilops squarrosa* (DD).

Female parent (AABB)	Male parent (DD)	Flowers pollinated	Grains formed (%)	Embryos rescued	Green plantlets	Fertile plants recovered after colchicine
1980						
T. dicoccum 22860	*Ae. squarrosa* 20229-C*	192	0.5	01		
T. dicoccum 22860	*Ae. squarrosa* 29445	96	2.1	02		
T. durum 22909	*Ae. squarrosa* 29229-C	544	0.7	04	04	04
T. durum 22912	*Ae. squarrosa* 20229-C	256	0.0			
T. durum 22909	*Ae. squarrosa* 29445	244	1.2	03	00	00
T. durum 22912	*Ae. squarrosa* 29445	448	0.4	02	00	00
		1,780	**0.7**	**12**	**4 (33.3%)**	**04 (100.0%)**
1981						
T. dicoccum 22844	*Ae. squarrosa* 29339	648	0.2	01	00	
T. dicoccum 22844	*Ae. squarrosa* 20211-Y	522	0.1	05	04	00
T. dicoccum 22844	*Ae. squarrosa* 20229-a	124	3.2	04	04	00
T. dicoccum 22844	*Ae. squarrosa* 20229-b	238	1.3	03	03	00
T. dicoccum 22844	*Ae. squarrosa* 20221	100	0.0			
T. durum 22912	*Ae. squarrosa* 20229-b	237	5.5	13	00	
T. carthilicum 20141	*Ae. squarrosa* 20229-a	144	8.3	12	00	
T. carthilicum 20141	*Ae. squarrosa* 20229-b	116	0.0			
T. carthilicum 20141	*Ae. squarrosa* 20291	16	0.0			00
		1,907	**2.0**	**38**	**11 (28.9)**	**0.0% (**)**
1982						
T. durum 22912	*Ae. squarrosa* 29342	2,110	2.5	53	38	38
T. durum 22912	*Ae. squarrosa* 29338	87	0.0			
T. durum 22912	*Ae. squarrosa* 20221	90	0.0			
		2,287	**2.3**	**53**	**38 (71.7%)**	**38 (86.8%)**
1983						
T. durum 44665	*Ae. squarrosa* 20211-C	332	0.0			
T. durum 44665	*Ae. squarrosa* 20211-Y	62	0.0			
T. durum 44665	*Ae. squarrosa* 29338	144	0.0			
T. durum 44665	*Ae. squarrosa* 29341	617	9.4	58	14	14
T. durum 44665	*Ae. squarrosa* 29381	456	0.0			
T. durum 22912	*Ae. squarrosa* 20211-C	108	6.5	07	06	06
T. durum 22912	*Ae. squarrosa* 20211-Y	200	5.5	11	05	05
T. durum 22912	*Ae. squarrosa* 20221-I	154	2.0	03	01	01
T. durum 22912	*Ae. squarrosa* 29341	615	2.0	11	07	07
T. durum 22912	*Ae. squarrosa* 29381	114	1.0	01	01	00
T. dicoccum 22860	*Ae. squarrosa* 20211-C	50	2.0	01	01	00
T. dicoccum 22860	*Ae. squarrosa* 20211-Y	158	0.6	01	01	00
T. dicoccum 22860	*Ae. squarrosa* 20221-I	266	1.5	04	02	00
T. dicoccum 22860	*Ae. squarrosa* 29338	86	0.0			
T. dicoccum 22860	*Ae. squarrosa* 29341	155	19.0	30	03	00
T. dicoccum 22861	*Ae. squarrosa* 20221-I	134	1.5	02	01	00
T. dicoccum 22870	*Ae. squarrosa* 20211	66	3.0	02	02	00
T. dicoccum 22870	*Ae. squarrosa* 20221-I	26	0.0			
		3,743	**3.5**	**131**	**44 (33.6%)**	**33 (75.0%)**
TOTAL		**9,717**	**2.4**	**234**	**94 (40.0%)**	**75 (79.8%)**

*Capital letters mean progeny of one selected plant. **Plants died before or in the beginning of tillering stage.

Table II - Results of crosses between *Aegilops squarrosa* (DD) and tetraploid species (AABB).

Female parent (AABB)	Male parent (DD)	Flowers pollinated	Grains (%)	Embryos rescued	Green plantlets	Fertile plants recovered
Ae. squarrosa 20211-C*	*T. dicoccum* 22860	64	20,0	13	03	0
Ae. squarrosa 20211-C	*T. dicoccum* 22870	288	21,0	61	05	0
Ae. squarrosa 20221-I	*T. dicoccum* 22860	42	24,0	10	00	
Ae. squarrosa 20221-I	*T. dicoccum* 22870	148	22,0	32	13	0
Ae. squarrosa 29341	*T. dicoccum* 22860	136	19,0	26	10	0
Ae. squarrosa 29381	*T. dicoccum* 22860	64	16,0	10	00	
Ae. squarrosa 20211-C	*T. durum* 22912	44	4,5	02	02	0
Ae. squarrosa 20221-I	*T. durum* 22912	54	7,0	04	03	0
Ae. squarrosa 29341	*T. durum* 22912	22	32,0	07	02	0
		862	**(19.1)**	**165**	**38 (23.0%)**	**0 (0.0%)****

*Capital letters mean progeny of one selected plant. **Plants died before or in the beginning of tillering stage.

Table III - Fertile new synthetic lines obtained from the crosses presented in Table I.

Line Number	Parental cross	Cross number
PF 804001	*T. durum* 22909/ *Ae. squarrosa* 20229-C	F 23800
PF 804002	*T. durum* 22909/ *Ae. squarrosa* 20229-C	F 23800
PF 834001	*T. durum* 22912/ *Ae. squarrosa* 29342	F 25992
PF 844001	*T. durum* 55559/ *Ae. squarrosa* 29341	F 25987
PF 844002	*T. durum* 55559/ *Ae. squarrosa* 29341	F 25987
PF 844003	*T. durum* 55559/ *Ae. squarrosa* 29341	F 25987
PF 844004	*T. durum* 22912/ *Ae. squarrosa* 20211-Y	F 25989
PF 844005	*T. durum* 22912/ *Ae. squarrosa* 20211-C	F 25988
PF 844006	*T. durum* 22912/ *Ae. squarrosa* 20221-I	F 25990
PF 844007	*T. durum* 22912/ *Ae. squarrosa* 29341	F 25991
PF 844008	*T. durum* 22912/ *Ae. squarrosa* 20221-I	F 25990

Consequently, the interaction between phytophatologists, agronomists, and cytogeneticists, similar to what is observed in wheat-exporting countries over the past 60 years, is extremely important for the improvement of bread wheat in South America in the near future.

The artificial resynthesis of hexaploid wheat is impossible to be done by conventional procedures. It is necessary environment-controlled support, cytogenetics and tissue culture techniques but, more critical than everything, it needs interdisciplinary cooperation to obtain useful products for breeding programs. It gives the possibility of obtaining homozygous hexaploid genotypes simultaneously combining useful genes from two species in one step.

The perspectives of molecular cloning-related genes and plant transformation increase the expectations to effectively harness the useful genetic variability identified in related species to improve yield potential and yield sustentability in wheat. The procedures here described certainly will remain basic for future experiments and meanwhile are more suitable for practical breeding.

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