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Plant breeding

Bread wheat x *Agrotricum* crosses as a source of immunity and resistance to the PAV strain of barley yellow dwarf luteovirus

A Comeau[1*], KM Makkouk[2], F Ahmad[1], CA Saint-Pierre[3]

[1]*Agriculture Canada, Station de Recherches, 2560 Hochelaga Blvd, Sainte-Foy, Quebec, G1V 2J3, Canada*[†] ;
[2]*Virology Laboratory, Genetic Resources Unit, International Center for Agricultural Research in the Dry Areas (ICARDA), PO Box 5466, Aleppo, Syria;*
[3]*Département de Phytologie, Université Laval, Sainte-Foy, Québec, G1K 7P4, Canada*

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Summary – The *Agrotricum* line OK7211542 ($2n = 56$) was immune to the Cloutier isolate, which belongs to the PAV serotype of barley yellow dwarf luteovirus (BYDV). Through a process of selection for BYDV resistance and threshable phenotypes with wheat-like seeds, wheat-like F_7-derived lines were obtained from *Triticum aestivum*\OK7211542 crosses. Some of these were immune to BYDV and had 44 chromosomes. Resistant lines with $2n = 42$ chromosomes or slightly higher chromosome numbers were also obtained. The low frequency of expression of immunity in the bread wheat background over many generations suggests that more than one gene is involved. These results are a first step towards the creation of wheat cultivars immune to BYDV. However, successful introgression will necessitate a transfer of immunity associated with a smaller amount of alien chromatin in the wheat genome.

introgression / *Thinopyrum* / *Lophopyrum* / BYDV / *Triticum*

Résumé – Croisements blé tendre x *Agrotricum* comme sources d'immunité et de résistance à la souche PAV du lutéovirus de la jaunisse nanisante de l'orge. *La lignée d'*Agrotricum *OK7211542 (2n = 56) est immune à l'isolat Cloutier, un isolat de sérotype PAV du virus de la jaunisse nanisante de l'orge (VJNO). Après une sélection pendant plusieurs générations pour la résistance au VJNO et pour un phénotype semblable au blé tendre quant à la facilité de battage et à la forme du grain, on a obtenu des lignées visuellement semblables au blé. Certaines lignées à 2n = 44 furent classées immunes au VJNO. Des lignées à 2n = 42 ou possédant quelques chromosomes de plus ont été classées résistantes au virus. La rareté des phénotypes immuns semblables au blé, au fil des générations, suggère que plus d'un gène est impliqué. Ces résultats sont une première étape vers la création de cultivars immuns au VJNO. L'introgression ne sera cependant complètement réussie qu'avec un transfert d'immunité associée à des quantités plus faibles de chromatine étrangère dans le génome du blé.*

***introgression* / Thinopyrum / Lophopyrum / *VJNO* / Triticum**

*Correspondence and reprints
†Contribution no 483.

INTRODUCTION

Barley yellow dwarf luteovirus (BYDV) is considered as the most damaging virus affecting small grain crops. The virus is aphid-borne, with 5 main pathotypes (PAV, MAV, RPV, RMV and SGV) differentiated essentially on the basis of their aphid vector specificity (Irwin and Thresh, 1992). BYDV disrupts the function of the phloem cells of numerous graminaceous species. Genes for immunity or high resistance to this virus are nonexistent in the genus *Triticum*.

The terms immunity, tolerance and resistance need precise definition. Immunity implies absence of virus reproduction and movement in the plant. A wheat line is called resistant if the virus multiplication or spread is reduced in comparison to susceptible checks. None of the *T aestivum* lines that we have verified with ELISA so far have fallen into this category; all were virus-invaded in less than 6 d. A line is called tolerant if it suffers less damage, despite similar virus spread and multiplication in its tissues when compared to the checks (Cooper and Jones, 1983). All the *T aestivum* sources of field tolerance to BYDV behaved in this manner (Makkouk, unpublished results). Tolerance genes can be very useful to plant breeders. However, the evaluation of tolerance faces the same difficulties as yield evaluation: it takes large plots, 2 or more years, and preferably many sites, as it is a quantitative trait (Qualset *et al*, 1973). Evaluation of immunity or strong resistance is very quick; tissue-blot immunoassay (TBIA) can give an answer about single plants in a matter of 3 weeks after seeding, 10 d after virus inoculation, and is not affected by spring or winter habits. Therefore the resistance and immunity genes are simpler to handle, but until now such genes were not available for bread wheat.

The lack of an acceptable level of BYDV resistance in the commercial bread wheat or durum wheat cultivars encouraged scientists to search for BYDV resistance in wheat wild relatives, to attempt a transfer of such resistance into the cultivated species. A number of cereal wild relatives such as *Thinopyrum* spp, *Agropyron* spp, and *Leymus* spp, have been reported to be either immune or highly resistant to one or more BYDV strains (Comeau and Plourde, 1987; Larkin *et al*, 1990). Attempts to transfer BYDV resistance genes from *Agrotricum* into wheat were initiated, often with the Zhong series of *Agrotricum* ($2n=56$), which was created in China from bread wheat x *T intermedium* (Chi *et al*, 1979; Brettell *et al*, 1988; Xin *et al*, 1988; Xin *et al*, 1991). Some of the taxonomical complexities of the genus *Agrotricum* and its wild genitors are discussed in an annex to this paper.

The line OK7211542 ($2n=56$, presumed to be *Agrotricum* amphiploid) has been reported as very resistant to BYDV (Cisar *et al*, 1982; Comeau *et al*, 1993a, 1993b). This line is evaluated in this study for its usefulness as a source of better BYDV resistance. The feasibility of obtaining advanced breeding lines of *T aestivum* with immunity or resistance gene(s) introgressed from OK7211542 is also assessed.

MATERIALS AND METHODS

Parental lines

The *Agrotricum* line OK7211542 ($2n=56$, exact genomes unknown) was chosen as the source of BYDV-PAV resistance as suggested by Cisar *et al* (1982). The high resistance was confirmed in our own trials (Comeau *et al*, 1993a), and by other groups (Banks *et al*, 1993). The exact pedigree of this line, derived from the previous interspecific hybridization program by Sando (Oklahoma, USA) in the fifties, was declared lost. The susceptible line of bread wheat was Yorkstar, a soft white winter wheat from the USA.

Breeding and selection methods

Conventional crossing was used to obtain 200 F_1 seeds of Yorkstar/OK7211542. From these, about 2 000 F_2 seeds of Yorkstar/OK7211452 were produced. A subsample of 10 F_1s of Yorkstar/OK7211542 was kept aside and inoculated with BYDV to evaluate gene expression in the hemizygous state. The F_2 plants were inoculated with the Cloutier BYDV-PAV isolate, using 10–15 viruliferous aphids per plant following a previously described method (Comeau, 1984; Comeau, 1992). To evaluate the frequency of F_2 plants that expressed resistance, 60 F_2 plants were grown in a separate trial to be compared with the parental lines Yorkstar and OK7211542 under BYDV-inoculation. The remainder of the F_2 plants was BYDV-inoculated in bulk. The 20% healthiest-looking F_2 plants were threshed vigorously, and the F_2-derived lots of F_3 seed were screened on 5/64 inch mesh. Seed lots made of narrow seed

types, similar to *Agrotricum*, were discarded. Only 40 seed lots were finally kept, bulked and advanced into further selection.

As the F_3 population thus selected was small, it was grown for 2 generations in bulk without artificial BYDV inoculation. In F_4 a small subsample of 10 wild and 10 wheat-like phenotypes was used for cytogenetic verification. From F_3 to F_5, the only selection pressure applied was from mechanical threshing, as the *Agrotricum*-like phenotypes were more difficult to thresh than the wheat-like phenotypes, and therefore more subject to mechanical elimination (Comeau *et al*, 1993a).

Roughly 40 000 F_5 plants were BYDV-inoculated. The 3% best-looking, on the basis of low symptoms and wheat-like phenotypes, were sampled at random. A subsample of 50 were labeled from B1 to B20 (wheat-like phenotype) and i1 to i30 (intermediate phenotype), and tested by ELISA (for resistance). The B11 line was kept for further study and crossing. The best material was re-bulked and amounted to about 10 000 F_6 seeds after caliber selection.

The F_6 plants were also inoculated with virus, and the 200 largest, healthiest-looking plants were tested with ELISA. Some more were also selected on the basis of low symptoms and good seed shape and caliber. Thereafter, only 75 F_6-derived individual spikes were kept to produce the F_7 generation. Head rows of the F_7 generation were artificially inoculated with BYDV to assess the uniformity of BYDV resistance. Fertility of spikes and seed caliber and shape were also evaluated. Two rows, labeled YOK 4 and YOK 19, were harvested as individual plants. Grain samples from these plants were labeled from YOK 4.1 to YOK 4.9, and from YOK 19.1 to YOK 19.12.

The TBIA was used to evaluate the virus resistance of these 21 F_7-derived F_8 lines, and of checks known for virus susceptibility (wheat) or immunity (*Agrotricum*). Seeds were fall-sown in the field and plants were BYDV-inoculated 10 d later. One month later, 6 plants per line were transferred to 10-cm pots and brought to the glasshouse. Testing for BYDV was done 6 times between October 28, 1992 and March 15, 1993.

ELISA procedures

The double antibody sandwich ELISA (DAS-ELISA) procedure of Clark and Adams (1977) was used in early trials. Leaf extracts were prepared by the Pollahne Extraction Press, using 1 ml extraction buffer per gram of leaves. ELISA plates were coated with 1 µg/ml immunoglobulin (IgG) and the dilution of the alkaline phosphatase-conjugated IgG used was 1:1 000. Both IgG and conjugate were obtained from Agdia (Indiana, USA). Samples were considered positive when their A_{405} absorbance value exceeded the healthy mean by 3 standard deviations (Sutula *et al*, 1986). In the F_5 evaluation, we used 2 replicates with 8 plants combined in each, we harvested plants 16 d after inoculation, and computed the ANOVA on ELISA values. The F_6 data was on single plants. Besides Yorkstar, susceptible checks included bread wheat cultivars Borden, Ruby and Glenlea.

The TBIA procedure was as previously reported (Hsu *et al*, 1990; Hsu and Lawson, 1991; Makkouk *et al*, 1993). Young or old stems were cut by a single-edge razor blade and directly blotted onto a nitrocellulose membrane (no BA85, 0.85 µm, Schleischer and Schuell). After 3 washes (3 min each) with phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20 (PBS-T), the membrane was incubated for 1 h in PBS-T containing 1% bovine serum albumin and 2% non-fat dry milk, followed by 3 washes with PBS-T. The membrane was then incubated for 1 h in BYDV antiserum produced in our laboratory against the PAV Cloutier isolate, diluted 1:1 000 in PBS. After 3 washes with PBS-T, the membrane was incubated for 1 h in anti-rabbit alkaline phosphatase conjugate (Sigma) diluted 1:2 000, followed by 3 washes as above. The nitro blue tetrazolium (NBT)/5-bromochloro-3-indolyl phosphate (BCIP) substrate was then added (10 ml/10 × 10 cm membrane) and the reaction was stopped after 10–15 min by washing the membrane 2–3 times with distilled water. The dark purple staining of phloem bundles containing virus was observed under a stereomicroscope at low magnification.

RESULTS

Response to BYDV-inoculation in parental lines

Leaf extracts from the parental wheat lines (sensitive) and OK7211542 (resistant) were made 3, 7, 13, and 18 d after virus inoculation. The DAS-ELISA A_{405} values increased steadily in the wheat lines, whereas there was no detectable virus in OK7211542 during the same period, according to the threshold (Sutula *et al* 1986) given by the mean plus 3 sd (fig 1A).

When BYDV invasion in the lines Yorkstar and OK7211542 was monitored by TBIA, results similar to those above were obtained. The difference between the 2 species was even more visible in this test (fig 1B). At 13 d after BYDV inoculation, around 33 stained phloem bundles were observed per cross-section of the wheat cv Yorkstar, whereas no

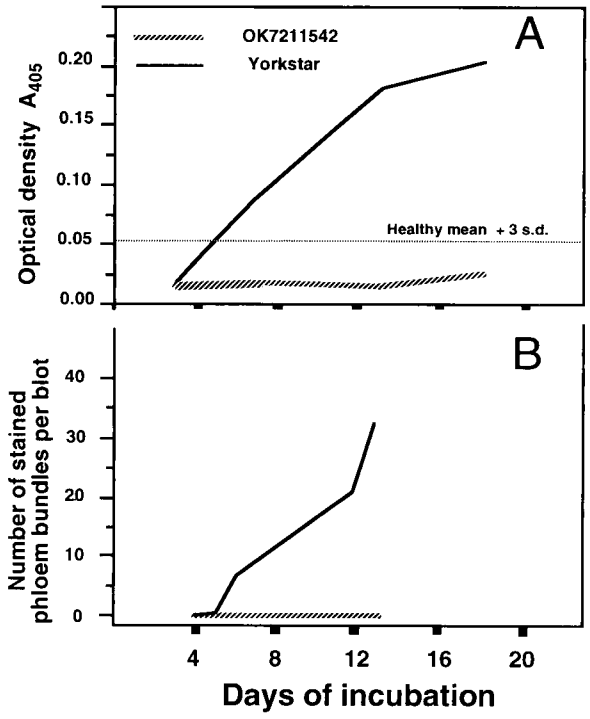

Fig 1. Detection of BYDV-PAV in leaf extracts by DAS–ELISA (A), and by tissue-blot immunoassay on nitrocellulose membrane (B) in winter bread wheat Yorkstar and an *Agrotricum*-derived line OK7211542 (average of 10 stem sections) for a range of incubation periods. After 150 d, Yorkstar had 20 stained bundles per section and OK7211542 still had none (not shown in fig 1B).

sign of virus presence was observed in OK7211542. The OK7211542 line continued to be virus-free, even at 150 d after virus inoculation and after overwintering and growth to maturity.

Effect of BYDV-inoculation on biomass in F_1 and F_2

When the *Agrotricum* OK7211542, Yorkstar bread wheat, and 10 F_1 of Yorkstar/OK7211542 were inoculated with BYDV after fall seeding, the *Agrotricum* remained green and vigorous, and averaged 147 g/plant for dry aerial biomass at maturity. Yorkstar suffered yellowing and its biomass was only 36 g/plant. The biomass for the F_1 was 81 g/plant, with yellowing present. The F_1 plants thus suffered significant virus damage, showing that the resistance gene(s) did not fully express in the hemizygous state in aneuploid F_1 plants.

The biomass of BYDV-inoculated segregating F_2 of Yorkstar/OK7211542 was also compared to that of parental lines. Most F_2 were diseased, like the sensitive parent Yorkstar, and suffered severe biomass reduction. A small percentage of the F_2 had biomasses that were better than those of Yorkstar plants (fig 2), but still had some visual symptoms, and complete resistance was absent in F_2. The OK7211542 plants did not have yellowing symptoms, despite some variation in biomass. Unevenly distributed winter stress may have interacted with aphid damage or fungal pathogens to cause biomass variation.

Effects of mechanical selection versus *BYDV selection*

The first cycle of BYDV selection caused retention of only 40 F_2 lines, intermediate between *Agrotricum* and wheat in phenotype. Mechanical selection pursued with the F_3 and F_4 seeds favored a gradual return towards wheat-like phenotypes. In a subsample of 20 F_4 plants of contrasting phenotypes, the 10 *Agrotricum*-like plants had $2n=56$ and were

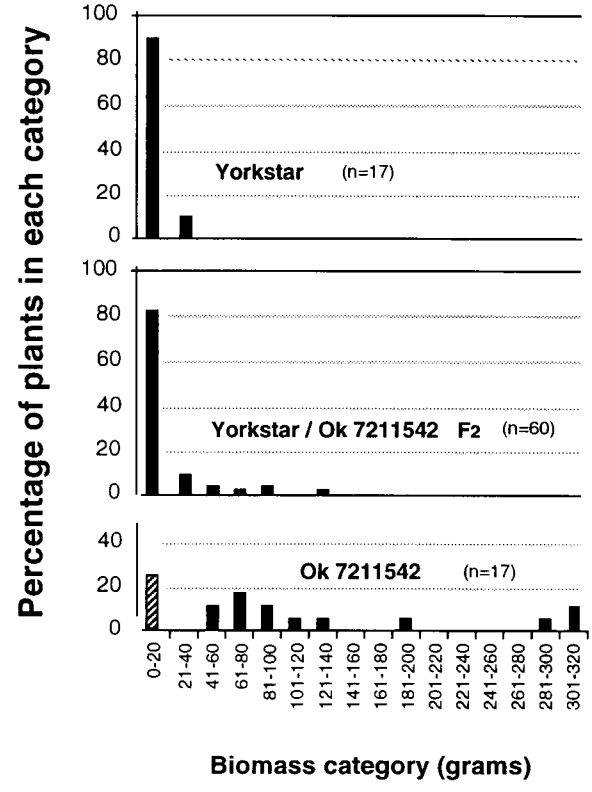

Fig 2. Frequency distribution of plants in various biomass categories in comparison with the parental lines with the F_2 population, under artificial BYDV-inoculation. The presence of plants in the 0–20 category for OK7211542 (▨) is accidental, being caused by winter kill.

mostly resistant, whereas the 10 bearing wheat-like spikes and seeds had from $2n=42$ to $2n=46$ and were rather diseased. This confirmed that the 2 selection pressures could orient chromosome numbers in opposite directions.

Resistance assessment in F_5–F_6

After the F_5 selection, no plant seemed immune but a small percentage of them looked rather resistant. In the subsampling of wheat-like (lines B1 to B20) and intermediate (lines i1 to i30) F_5-derived lines, ELISA values differed between genotypes ($P< 0.01$). Line B11 ($2n=42$, A_{405} = 0.040) and line B3 ($2n=44$, A_{405} = 0.045) had low ELISA values together with a wheat-like phenotype. Lines i27 and i12, of a slightly wild phenotype with long internodes in the spike, were $2n=42$ and had low ELISA values of 0.057 and 0.063, respectively. Other lines in that range of ELISA values had more chromosomes (44–46) and more wild traits. Some lines had a wheat phenotype with high ELISA value, such as line B8 with $2n=42$ and ELISA of 0.868, which was higher than that of the susceptible check bread wheat Glenlea at 0.305. All the 50 lines were positive for BYDV, using the threshold of 0.024 (mean plus 3 sd). Immunity was thus not found.

The 75 F_6-derived lines kept had either the least visual symptoms or the lowest ELISA, or both. Progress towards resistance became quite visible.

Evaluation of F_7 and F_8 headrows

The 75 lines were first evaluated by symptoms, and it was obvious that many plants were visually similar in BYDV resistance to OK7211542. Two complete rows (YOK4 and YOK19) had fertile wheat-like seeds and spikes, with uniform high resistance. Spikes were taken from these to make the 21 F_8 headrow reselections. The lines were almost but not totally fixed for morphological traits, despite the advanced generation level. Virus presence in the 21 selected F_8 lines was evaluated by TBIA. The 21 F_8 lines were thus classified into 3 categories.

Group 1: 5 lines (YOK 4.9, YOK19.2, YOK19.5, YOK19.6, and YOK19.7) were immune to infection, as no virus was detected in any of the tests.

Group 2: 9 lines (YOK4.1, YOK4.3, YOK4.4, YOK4.5, YOK4.6, YOK4.7, YOK4.8, YOK19.11, and YOK19.12) were highly resistant, as small traces of virus infection was detected in some tests, and plants were able to fully recover, since the virus was not detected at maturity.

Group 3: 7 lines (YOK4.2, YOK19.1, YOK19.3, YOK19.4, YOK19.8, YOK19.9, YOK19.10) were resistant, as only a moderate number of infected phloem bundles were detected, at least once during the virus-testing period. However, these lines were visibly much more resistant than other comparable winter wheat lines present in the same trial, namely Borden and Ruby, which had heavy phloem infection at all sampling dates. Tissue-blots of stem sections of plants representing immune and moderately resistant genotypes, in addition to the original parents Yorkstar and OK7211542, are shown in figure 3.

Chromosome counts were obtained on 16 plants from 8 of the 21 lines, representing the 3 BYDV categories at the F_8 level. All plants studied showed a diploid chromosome number of $2n=44$ (fig 4). These may be addition lines with 42 chromosomes of wheat and 2 from the alien species, but other possibilities could be envisaged. In line YOK4.9, there were plants with $2n=44$ complete

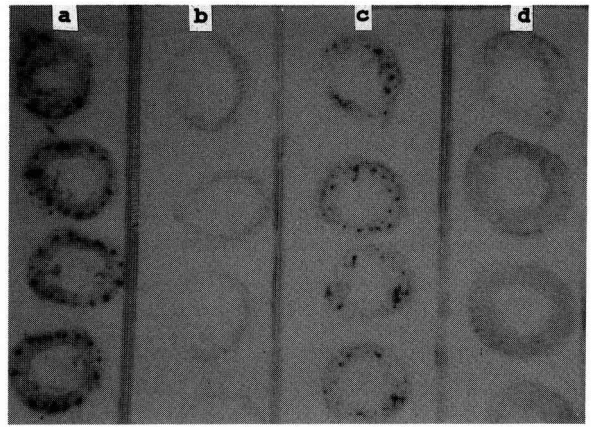

Fig 3. Tissue-blots of stem sections of (a) Yorkstar, (b) OK7211542, (c) a plant of one of the F_8 lines in category 3, and (d) a plant of one of the immune F_8 lines of category 1 (see text).

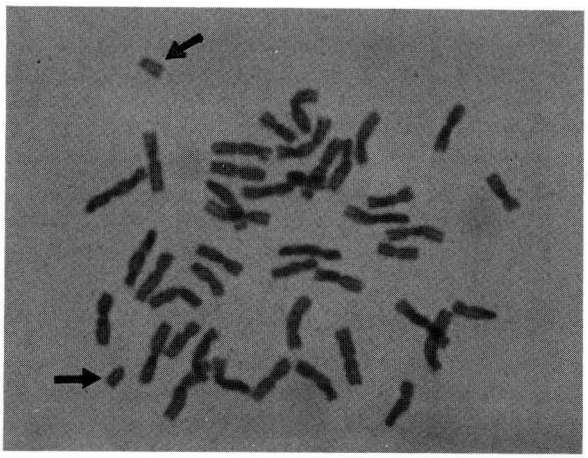

Fig 4. A root tip cell from a plant from one of the BYDV immune lines, YOK 4.9, showing $2n=44$ chromosomes (42 complete and 2 telocentric). Arrows indicate the telocentric chromosomes.

chromosomes and also plants with 42 complete plus 2 telocentric chromosomes (chromosomes with only 1 arm).

DISCUSSION

The first goal of this work was to transfer to a wheat-like plant the immunity to BYDV-PAV that exists in the *Agrotricum* line OK7211542.

The level of resistance obtained in some $2n=44$ F_7 lines (group 1 above) deserves to be called immunity. Other lines must be called highly resistant rather than immune (group 2 above), because of the presence of a few infected phloem vessels at one sampling date during the plant life cycle. Yet these group 1 and group 2 lines had a level of resistance higher than a number of the *Agrotricum* lines For example, the *Agrotricum* line Zhong 5 and its derivatives are called resistant, not immune, as they contain measurable amounts of virus (Xin *et al,* 1991), which was also confirmed by TBIA (Comeau, unpublished results). In contrast, no evidence of virus multiplication was found in OK7211542 at any sampling stage.

The antagonistic effects of the 2 selection pressures, firstly virus resistance selection and secondly mechanical seed caliber and threshability selection, were not unexpected. The combination of the 2 selection pressures gradually increased the frequency of the occasional plants which presented a compromise in phenotype, with some resistance and some wheat-like traits. Such plants would frequently be aneuploid and partly sterile, as they had to possess relatively few alien genes, but enough to behave somewhat like OK7211542 under virus infection. Even in F_5, the immunity could not be found in lines of wheat-like phenotype: the B11 selection was resistant, not immune. The desired result was only demonstrated in F_7. Natural cross-pollination within the still segregating, partly male-sterile resistant bulk population may have been useful in creating YOK4 and YOK19, which contained immunity and near-immunity.

The immunity trait of OK7211542 did not behave as a monogenic factor, as it would have most likely shown up in F_2 despite non-mendelian segregation. The number of genes involved remains unknown, and is under investigation. The lower resistance levels found in F_5 (as in line B11, $2n=42$) gives extra clues about genetic complexity. The immune $2n=44$ lines are likely to contain the resistance of B11 plus some other gene(s). The presence of 2 extra chromosomes in selected F_8 lines indicates these may contain key elements responsible for immunity or high resistance.

Additional chromosomes may be lost in later generations due to addition decay. Hence the resistance genes from the additional chromosomes must be transferred to wheat chromosomes. Another backcross to wheat would increase the odds of homoeologous recombination. The observed telocentrics were the product of chromosome cleavage, indicating a possibility for eventual centric break-fusion which could lead to the desired $2n=42$ wheat.

More strains of BYDV should be used to verify the range of protection conferred by the alien genes. Introgression of the immunity genes of the F_7–F_8 derived lines into the wheat genome through the use of pairing promotors such as ph1b would be a logical route. This approach is sound especially as the trait seems to be controlled by more than one alien gene. Pollen irradiation-induced translocations should also be attempted. Work in this area is now underway. The movement of small amounts of alien chromatin on and between chromosomes could be monitored by fluorescent *in situ* hybridization (FISH), RAPD and RFLP analysis.

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Annex – Taxonomical considerations about *Agrotricum*

There is a vast body of literature from USSR, USA and Europe dealing with the *Agrotricum* genus, derived from crosses of the former genus *Agropyron* with wheat. It can be therefore considered as accepted by practice. However, this comes with a warning that the genomes of current *Agrotricum* lines are generally unknown, and may be of segmental nature or very different between 2 different accessions of this genus. Also, the former genus *Agropyron* has undergone many revi-

sions. The species *Thinopyrum ponticum* and *T intermedium* were formerly classified under *Agropyron* or *Elymus,* according to various taxonomists. The *Thinopyrum* genus itself was created in 1980. Within the last 15 yr, the decaploid *Agropyron elongatum* 10x successively became *Elytrigia elongata*, then *Thinopyrum ponticum*, and more recently it was reclassified by some authors as *Lophopyrum ponticum*. A diploid species previously known as *Agropyron elongatum* 2x became the type species for this revision of the genus *Lophopyrum*. Parallel changes at the genus level were made for the hexaploid species *Agropyron intermedium* (syn *Elymus hispidus* or *A glaucum*), which was at one point called *Thinopyrum*, then *Elytrigia*, and recently *Lophopyrum*. Further reclassifications may be attempted as the genomes of these genera become better known. According to current rules used to name synthetic species, *Agrotricum* (syn *Agrotriticum*) could therefore have become *Elymotriticum, Thinopyrotriticum, Elytrigitriticum,* and now *Lophopyrotriticum*. We thought that in such circumstances the *status quo* was temporarily acceptable for the genus *Agrotricum.* Although a recent monograph declares *Thinopyrum* should still be called *Elymus* (Clayton and Renvoize 1986), we preferred to retain the terminology proposed in the global revision of Dewey (1984), who described the taxonomical history of perennial Triticeae and based his reclassification on the genomic analysis of interspecific hybrids.

The origin of the *Agrotricum* line OK7211542 is also subject to a word of caution. In a recent report it was said to be a derivative from *T intermedium* (Banks *et al,* 1993). This needs further investigation. We had requested such information from Sebesta and Smith at Oklahoma State University and obtained the answer that the exact pedigree was no longer available.

A private report written by Sando himself in the mid-fifties about his interspecific collection mentioned 670 *Thinopyrum ponticum* derivatives, and only 31 lines from *T intermedium*. Moreover, in Sando's own words, the wheat x *T ponticum* hybrids were "interesting sources of resistance to rust, mildew, septoria blotch, streak virus mosaic, hessian fly, jointworm and aphids". He made no comments about the resistance of his hybrids with *T intermedium*. In those days, BYDV was barely known, and, although aphids can be damaging, heavy losses more often come from the BYDV transmitted by the aphids. Therefore, what Sando reported as aphid resistance could involve BYDV resistance or immunity.

Another piece of circumstantial information led us to hypothesize that OK7211542 may have *T ponticum* ancestry. In 1981–86, we created a number of wheat x alien derivatives with both *T ponticum* and *T intermedium*. Progenies were selected with BYDV-inoculation during the selfing generations after backcrossing. The end result with wheat x *T intermedium* was never immunity. At best, a potentially useful level of resistance similar to that of Zhong 5 (previously mislabeled as Zhong 4, see Banks *et al*, 1993) was obtained in a few lines.

However, from the crosses to *T ponticum* cv Orbit, we obtained 7 BYDV-PAV immune amphiploids, as evaluated by TBIA. These new lines had a strong morphological resemblance to OK7211542. Many had $2n=56$; some are still unstable. As *T intermedium* and *T ponticum* have some genomes in common, solving the enigma of the origin of OK7211542 may be difficult. Nevertheless, we could produce lines similar to OK7211542 using *T ponticum*, and not with *T intermedium*. This reinforces the case in favor of a *T ponticum* origin.

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