The expression of modified rye ribosomal RNA genes in a wheat background

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[1]Contribution of USDA-ARS and the Department of Agronomy, University of Missouri, Columbia, MO 65211. Financial support provided by United States Department of Agriculture Competitive Research Grant No. 84-CR-1-1412.

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ABSTRACT

C-banding, which stains the heterochromatic regions of chromosomes, was utilized in order in ascertain whether or not several varieties of wheat (Triticum aestivum L. em Thell.) contain a 1R(1B) substitution or a 1RS/1BL translocation. In many of the wheats containing an observable rye (Secale cereale L.) segment, the C-band corresponding to the nucleolus organizing region (NOR) was significantly reduced. Therefore, to determine if a decrease in the size of the NOR C-band had any effect upon the expression of the rye ribosomal RNA genes which are contained within the NOR band, in situ hybridizations of root-tip cells with a biotin-labeled rye NOR probe were carried out. Since genes which are being actively transcribed are dispersed and those genes which are inactive remain condensed, this procedure is a simple method for analysing for gene expression by scanning prepared slides for interphase nuclei and comparing label that is either condensed or dispersed. None of the substituted or translocated wheats which contained the reduced NOR locus showed any rye rDNA expression, suggesting that the number of rye ribosomal genes and/or spacer units is very important for any degree of rye NOR activity when the rye is present in a wheat background.

Could you show activity in which the NOR was not reduced?

Substitutions of rye (Secale cereale L.) chromosomes into wheat (Triticum aestivum L. em. Thell.) backgrounds, or translocations involving wheat and rye chromosomes in wheat backgrounds, have been reported by many authors. Mettin et al. (1973) and Zeller (1973) showed that a 1RS/1BL translocation is present in two well-known Russian varieties, Aurora and Kavkaz. These two varieties were used in many wheat-breeding programs around the world, and the 1RS/1BL translocation has been transmitted to a number of varieties (Blüthner and Mettin, 1977). According to Rajaram et al. (1983) and Zeller and Hsam (1983), varieties carrying this translocation have many advantages, including increased yield. Also, since this rye segment is known to carry several genes for disease resistance, selection for resistance in progenies from crosses involving Aurora or Kavkaz as one of the parents has favored this translocation.

Many wheat varieties from around the world have been derived from Aurora and Kavkaz and it could be expected that a 1RS/1BL translocation would be present in these varieties as well. Electrophoretic analyses indicated that most of them contained rye protein, and the conclusion was reached that these varieties carried the 1RS/1BL translocation. However, electrophoretic techniques can only analyse for the expression of a protein from a particular gene and do not necessarily reveal the presence of a particular chromosome or chromosome arm. Therefore, the wheats were analyzed using C-banding in order to ascertain whether or not these wheats contain a 1R(1B) substitution or a 1RS/1BL translocation.

During the analysis it was observed that the C-band corresponding to the nucleolus-organizing region (NOR) present on the short arm of rye chromosome 1R was much smaller than the same region present in the

parents used to create the wheat varieties. The deletion was very similar in appearance and size to that observed by Brettell et al. (1986) which they correlated with a significantly reduced number of ribosomal DNA spacer sequences. Because the results of Appels et al. (1986b) indicated that the rye rDNA is functional part of the time, even in a wheat background, it was of interest to ascertain the possible expression of the rDNA in the wheats which had a majority of their rye NOR deleted. Since a dispersed state of rDNA is known to be highly correlated with nucleolus formation and transcription into ribosomal RNA (Flavell et al. 1986), an analysis of the wheats using a rye rDNA spacer probe by in situ hybridization was utilized in order to detect rye NOR activity.

MATERIALS AND METHODS

Several wheat varieties bred at the Institute of Field and Vegetable Crops, Agricultural Faculty, University of Novi Sad, Yugoslavia were utilized because a wide range of parents was involved in producing these varieties. In addition, Snoopy rye, from the International Maize and Wheat Improvement Center (CIMMYT), Mexico, one triticale (x Triticosecale Wittmack) line NS TR 37 from Novi Sad, plus a few well-known standard wheat varieties were also analyzed. The Novi Sad varieties were Balkan, Duga, Jugoslavija, Kozara, Licanka, Macvanka 2, Novosadska 100, Novosadska Rana 1, Posavka 1, Posavka 2, Sava, Somborka, Sutjeska, Zelengora, Zvezda and Zitnica. The standard wheats were Anza with a 1R(1D) substitution, Aurora, Chinese Spring, Glennson (1RS/1BL), Gohls 121 and Kavkaz.

The 134 bp tandemly repeated TaqI spacer fragment, pScR4-T1, which was isolated from the rye insert pScR4 as described by Appels et al. (1986b), was used for the in situ hybridization experiments.

To test for the presence of a 1RS/1BL translocation or a 1R(1B) substitution, several different methods were employed:

C-banding. This method used was described by Bennett et al.(1977) and modified by Lukaszewski and Gustafson (1983). All varieties mentioned above were included in this study. For each variety a minimum of 6 different plants and 10 different cells per plant were analysed.

SDS-PAGE of unreduced proteins. Unreduced proteins were extracted from flour samples with .0625M Tris (pH 6.8), 10% (v/v) glycerol, 4% (w/v) SDS and .001% (w/v) Bromopenol Blue and electrophoresed using the protocols

of Singh and Shepherd (1985). The gels contained 10% acrylamide and were 1.5mm thick. Each gel was run at 40mA until the dye front escaped the stackng gel, then at 25mA for the remainder of the run. Of the varieties tested by C-banding, 13 were selected for analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Included were Snoopy rye, triticale NS TR 37 and 11 wheat varieties: Chinese Spring, Glennson, Anza 1R(1D), Duga, Sava, Kavkaz, Zvezda, Balkan, Posavka 2, Jugoslavija, and Zitnica.

In situ hybridization. For biotin labeling, the rye probe pScR4-T1, developed by R. Appels, CSIRO, Canberra, Australia, which is specific for the rye NOR locus, was used with the technique described by Rayburn and Gill (1985) with the following modifications. Biotinylated dUTP was used to nick-translate 1.5ug of probe DNA for only 2 hours at 15°C. Also, the hybridization mixture contained 40ug of carrier DNA for every 1ug of nick translated probe in a solution of 50% formamide, 10% dextran sulfate and 2XSSC.

Cytological preparations were made by placing mitotically synchronized root tips in ice-water overnight to condense their chromosomes. They were then treated with 45% glacial acetic acid for 15 hours, followed by 1 hour in 0.2N HCl. The roots were squashed using 45% glacial acetic acid and the slides placed on dry ice to remove coverslips. Slides were then air-dried and stored desiccated overnight. Storing the preps longer than overnight at room temperature or at -70°C impaired detection; therefore only freshly prepared slides were used. The varieties selected for biotin labeling were Balkan, Jugoslavija, Posavka 1, and Posavka 2 because these wheats possessed a reduced NOR

locus. Also, triticale line NS TR 37 and Glennson were used because these varieties contained a normal sized NOR in addition to Sava, an unsubstituted wheat, and Snoopy, an unsubstituted rye. In this experiment between 4 and 6 different plants were studied and several hundred cells per plant were scored.

RESULTS AND DISCUSSION

C-banding analysis

Several different observations were made for each of the varieties listed. C-banding patterns of metaphase chromosomes were studied to identify individual chromosomes. Interphase and prophase cells were analyzed for the presence of large blocks of the telomeric heterochromatin which is characteristic of rye chromosomes. Also, the numbers of satellite chromosomes per cell were determined to establish the number of active NOR loci, which are known to create a secondary constriction whenever they have been actively transcribed.

In the varieties where 4 satellite chromosomes were present, there was no rye heterochromatin detectable in interphase or late prophase cells and no metaphase chromosome had the characteristic 1R C-banding pattern. In the varieties where only 2 satellite chromosomes were seen, rye heterochromatin was always observed in the interphase and late prophase stages. When these varieties were analysed at metaphase, two homologous chromosomes had the rye C-banding karyotype of 1R (Fig 1A).

In the Novi Sad varieties Duga, Novosadska Rana 1, Sava, Somborka and Zitnica, C-banding could not detect any rye segments. This agrees with previously known results for the varieties Sava and Novosadska Rana 1. These two varieties are cytologically well-known, and have the same chromosome structure and pattern as the variety Chinese Spring, in that there are no translocation differences.

The Novi Sad varieties Balkan, Jugoslavija, Kozara, Licanka, Macvanka 2, Posavka 1, Posavka 2, Sutjeska, Zelengora and Zvezda each contained a 1RS/1BL translocation detected easily using C-banding. Variety Novosadska 100 had a 1R(1B) substitution. All of the above varieties

containing either a rye translocation or substitution had a significantly smaller NOR C-band than the rye NOR C-band present in the standard varieties (e.g., Glennson and Anza 1R(1D)). The Novi Sad wheat varieties contained approximately an 80% deletion of their rye NOR locus (Fig 1B). Also, the long arm of chromosome 1B in these wheats had fewer interstitial C-bands than the 1BL present in Chinese Spring.

The variety Neuzucht, known to possess a 1R(1B) substitution, was one common parent to Skorospelka 35, Aurora and Kavkaz. Skorospelka 35, which was characterized as having only two satellite chromosomes by Blüthner and Mettin (1977), could possibly have been the source of rye genes in Balkan, Posavka 1 and Posavka 2, since it is the only variety common in the lineage of all of these wheats, each of which contains a 1RS/1BL translocated chromosome. Jugoslavija, Kozara, Licanka, Sujeska and Zelengora are likely to have received their 1RS/1BL translocation from Aurora, since this is the only common parent of these varieties believed to contain rye genetic material. The other two Novi Sad varieties, Macvanka 2 and Zvezda, probably inherited their rye from Kavkaz, a parent common to both.

The source of the 1R(1B) substitution found in Novosadska 100 is difficult to determine, since none of its parents analysed to date contains a rye segment. The German variety Gohls 121 was speculated to be the rye donor, but cytological examination of its chromosome constitution in the present study did not reveal any rye heterochromatin, nor were any satellites visible. Therefore, the source of the 1R(1B) substitution in Novosadska 100 was probably due to an uncontrolled outcross.

Unexpected results were found for the varieties Aurora and Kavkaz.

In both, 4 satellited chromosomes were consistently observed and rye heterochromatin was not visible in any stage. No evidence of rye chromosome segments could be seen using C-banding. Since these varieties have been well documented as containing a 1R/1B translocation, these lines will be analysed again, with seeds taken from known seed sources at the Novi Sad Experimental Station.

SDS-PAGE of unreduced proteins

SDS-PAGE was used to study further the unexpected results from C-banding. Because the presence of chromosome arms 1BS and 1RS was in question, SDS-PAGE of unreduced total protein extracts was utilized. With this procedure, bands corresponding to Gli-B1 proteins, which are coded by 1BS, as well as the rye storage proteins, Sec-1, coded by genes on 1RS, can be easily identified (Koebner and Shepherd, 1986; Singh and Shepherd, 1985).

The electrophoretic patterns in Fig. 2 clearly show the presence of Gli-B1 proteins in Chinese Spring, Anza 1R(1D), Triticale NS TR 37, Sava, Kavkaz and Zitnica, although with differing intensities probably due to varying protein concentrations in the seeds of each variety. Since the genes for these protein bands have been mapped to chromosome arm 1BS (Shepherd, 1968), these varieties must contain at least a portion of this chromosome arm. The fact that Kavkaz was among the varieties expressing the Gli-B1 proteins strengthens the C-banding evidence that the expected 1RS/1BL translocation is missing from this sample of the variety.

Also in Kavkaz, there are no bands corresponding to Sec-1 proteins. Since these subunits are coded by genes on rye-chromosome-arm 1RS (Shepherd and Jennings, 1971), it is not unreasonable to suggest that this arm is absent in the Kavkaz seed stock analyzed. Only Snoopy rye, Glennson (1RS/1BL), Anza 1R(1D), Triticale NS TR 37 and the Yugoslavian wheats Zvezda, Balkan, Posavka 2 and Jugoslavija contained bands corresponding to Sec-1 proteins. All of these results agree with the C-banding analysis.

The expression of rye rDNA in Yugoslavian wheats

In rye and other cereals containing the rye chromosome 1R, the dispersion of active rye rDNA in nuclei, as assessed by *in situ* hybridization techniques, has been established by Appels *et al*. (1986b). They stated that this dispersed state is correlated with nucleolus formation because either transcription of rDNA results in dispersion (Flavell *et al*., 1986; Hilliker and Appels, 1982; Sommerville, 1985) or the dispersion is a result of rDNA undergoing replication (Barlow, 1984). Although the latter possibility is hard to dispute, there is no convincing evidence that replication could lead to such a high degree of dispersion. Finally, the nuclei analysed in the present study were picked so that they would likely be in either G1 or G2 and thus eliminate the S phase, which is where replication is believed to occur.

The biotin-labelling technique developed for plants by Rayburn and Gill (1985) gave much cleaner results than the classical *in situ* hybridization technique used by Appels *et al*. (1986b), in that silver-grain dispersion and/or background noise and the variation from slide to slide were eliminated. With biotin labelling, the color was either condensed or not, and the slides were very clean and easy to analyse.

When Snoopy rye was analysed, approximately 80-90% of all nuclei showed that at least one of the 1R's was in the dispersed state and thus was being actively transcribed (Fig. 3). This is in complete agreement with the results observed by Appels *et al*. (1986b). The present results also confirm that the cells of a rye root tip are highly heterogeneous and do not appear to utilize their rDNA to the same degree, or at the same stage of root development.

Because the rye NOR spacer probe shows some degree of cross hybridization with the wheat NOR spacer loci, it was possible to see some reaction when the wheat varieties were labelled with the rye rDNA probe (Fig. 4). However, this reaction was never observed to occur with the same intensity as in diploid rye. The hybridization sites observed in wheat were always very small, indicative of the limited cross reaction between the rye rDNA spacer probe and wheat.

An analysis of wheats containing either a 1B(1R) substitution or a 1RS/1BL translocation and of triticale NS TR 37 revealed that the rDNA spacer probe labels these alien rye NOR loci as it would label diploid rye NOR loci. When the rye NOR was inactive, two large blocks of label were clearly observed; and when the rye NOR was active, the label was dispersed throughout the nucleus. In most cases the dispersion was located near the nucleolus. In the wheats and the triticale that did not contain any deletion of their rye NOR locus, approximately 90% of nuclei contained two inactive rye NOR's; however, there were a significant number of nuclei that showed some degree of rye rDNA dispersion (Fig. 5A). These results agree with those observed by Appels et al. (1986) in that some measure of rye NOR activity is discernible even when in a wheat background.

When the 1B(1R)-substituted or the 1RS/1BL-translocated Yugoslavian wheats, which contain approximately an 80% deletion of the rye NOR region, were analysed, a totally different picture of rDNA expression was seen. None of the Yugoslavian substituted or translocated wheats showed any rye rDNA expression (Fig. 5B). The activity of the deleted rye NOR locus was always suppressed in the wheat backgrounds analysed. When compared to the other wheats analysed in the present study showing no rye

NOR deletion and those analysed by Appels et al. (1986b), the present results suggest that the amount of rye rDNA spacer units present in a wheat background is very important, even for some degree of limited activity. This agrees with the results of Flavell et al. (1986) which showed that gene expression can be positively affected by the number of "regulatory" sequences that lie 2000 to 3000 base pairs upstream from the rDNA promoter.

As stated in Appels et al. (1986b), the importance of the rDNA spacer in situations where rDNA units are actively competing for a defined pool of RNA polymerase I, and/or other factors required for initiating transcription, is becoming clear. In Xenopus laevis (Moss 1983; Reeder et al. 1983) an rDNA unit containing a spacer with more repetitive units is transcribed more often, in a competitive situation, than one containing fewer spacer units. This also appears to be the case in wheats (Appels and Dvorak 1982; Martini and Flavell 1985) and wheats containing rye chromosome 1R or the short arm of 1R (Appels et al. 1986b). Flavell et al. (1986) have expanded this by suggesting that the sequences upstream from the ribosomal DNA promoter increase gene activity by binding specific transcription-enhancing proteins. Therefore, the most abundant and effective regulatory sequences will consume the limited supply of enhancer protein and thus help determine which rDNA will be transcribed. Because the rye NOR locus contains shorter rDNA spacer units than those of wheat (Appels et al. 1980), it is the rye NOR which is suppressed or partially suppressed in a wheat background. In the Yugoslavian substituted or translocated wheats the rye NOR locus not only contains shorter rDNA spacer units than wheat but the total number has been decreased as a result of the deletion. It appears that the

combination of rye rDNA spacer unit size and copy number present in the Yugoslavian wheats analysed has resulted in the permanent suppression of all rye rDNA activity.

Acknowledgements.

Dr. S. Petrovic wishes to thank the Fulbright Program for the award of a Grant during which this work was undertaken. We are all thankful to Dr. E.R. Sears for his critical review of the manuscript, and to Dr. R. Appels for donating the rye nucleolar organizer probe.

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Fig. 1. A comparison of the C-band at the NOR site of A. chromosome 1R from the Yugoslavian wheat, Novosadska 100 and B. chromosome 1R from triticale NS TR 37.

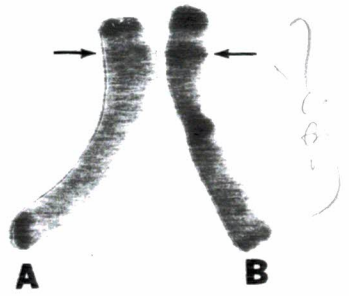
Fig. 2. SDS-PAGE pattern of unreduced protein samples indicating bands corresponding to Gli-B1, controlled by wheat chromosome arm 1BS, and the Sec 1 proteins, which are controlled by rye chromosome arm 1RS. 1. Chinese Spring, 2. Glennson 1RS/1BL, 3. Anza 1R(1D), 4. NS TR 37, 5. Duga, 6. Sava, 7. Kavkaz, 8. Zvezda, 9. Balkan, 10. Posavka 2, 11. Jugoslavija, 12. Zitnica, 13. rye var. Snoopy.

Fig. 3. Interphase nucleus of rye, var. Snoopy, showing dispersion of label, thus indicating activity of NOR genes.

Fig. 4. Chromosomes of A. 1R, B. wheat, and C. interphase nucleus of Glennson, showing cross-reaction of rye NOR probe with wheat NOR areas.

Fig. 5A. Interphase nucleus of Glennson showing acitvity of NOR on 1R. 5B. Typical interphase nuclei of the Yugoslavian wheat, Posavka 2, showing condensation of label, indicating no activity of rye NOR genes.

Fig 1



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IR Yugoslav Lothian

IR for tritical

fig 2

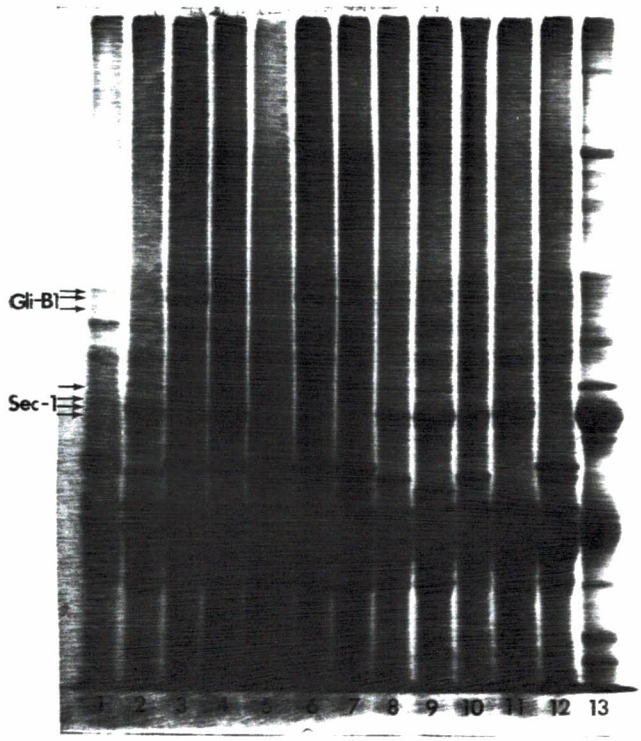
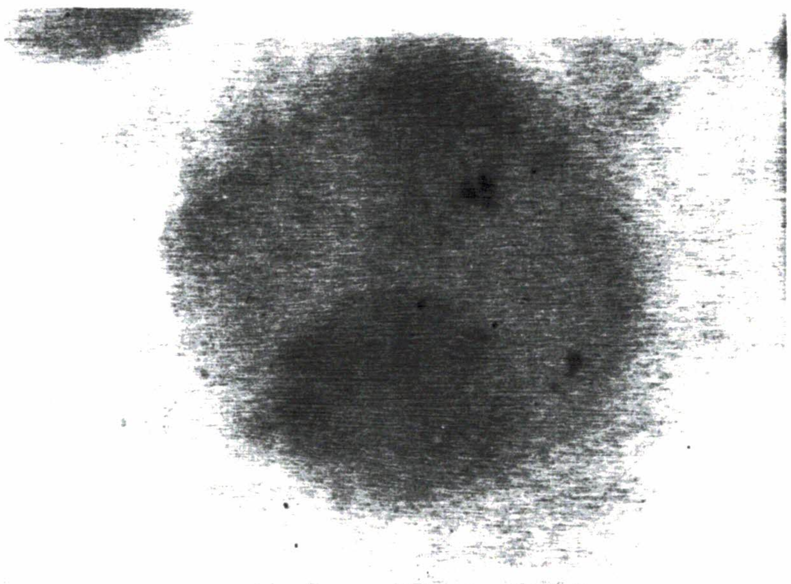


fig 3



Ring

Nuclear Ring on Spread

dispersal of label of nick translation at [illegible]

fig 4

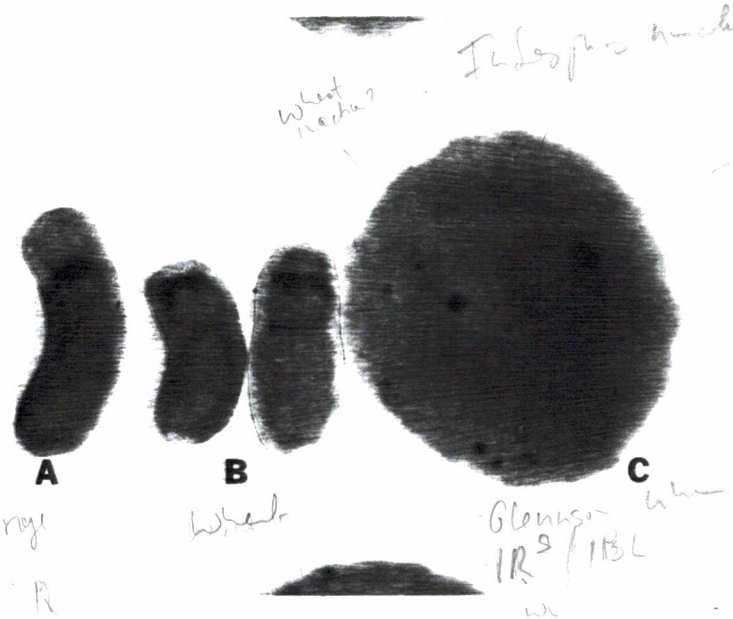


fig5