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LOCATION OF MARKERS OF ALUMINIUM TOLERANCE GENES ON RYE CHROMOSOMES (*SECALE CEREALE* L.)

ABSTRACT

The aim of presented work was to identify of PCR amplified DNA fragments differentiating aluminium tolerant and sensitive forms of rye and to locate the markers on rye chromosomes. For identification of markers, the PCR system with semi-specific primers targeting intron-exon sequences of plant genes was applied. The modified method of bulked segregant analysis was used. The pooled DNAs of two or three F_2 segregating populations were screened together with DNA of their parental inbred lines. Potential marker of tolerance gene was located on rye chromosomes using wheat/rye (Chinese Spring/Blanco) additional lines. The specific probes obtained from DNA fragments differentiating sensitive and tolerant forms of rye were hybridized to PCR amplified DNA fragments of sensitive and tolerant forms of rye and the set of wheat/rye addition lines. Independently of the method of digoxygenin labelling (primer extension or Taq polymerase reaction), the probes obtained showed similar hybridisation patterns. The results of hybridisation of 21 probes prepared from 12 DNA fragments confirmed connection of selected DNA fragments with to aluminium tolerance or sensitivity. Most of these DNA fragments originated from tolerant forms of rye. Using this method it was possible to locate eight DNA fragments on rye chromosomes. Three DNA fragments hybridised to chromosome 4R, two DNA fragments to chromosome 6R and single DNA fragments to chromosomes 1R, 2R and 3R. Four DNA fragments indicating clear relationship with character studied were not located on particular chromosomes using this set of wheat/rye addition lines. Hybridisation of probes prepared from four DNA fragments revealed length polymorphism. Probes prepared from two DNA fragments were characterised as dominant markers. In other cases the type of marker (dominant/codominat) was not fully documented.

Key words: aluminium tolerance, chromosomal location, DNA-DNA hybridisation, PCR markers, wheat/rye addition lines

INTRODUCTION

Aluminium is the most abundant metal in the soils. At neutral and weakly acidic pH aluminium exists in the form of insoluble minerals and aluminium ions are not available for growing plants. When soil becomes more acidic naturally or as the result of human industrial activity and mineral fertilisation, part of minerals is solubilized into toxic trivalent cations available to plants. It is estimated that acidity affects about 40% of world arable land. The first visible effect of aluminium toxicity is the inhibition of root elongation by blocking the division and elongation of cells at the root tip. This causes the impaired uptake of nutrient and water and subsequently results in decrease

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of plant growth and productivity. In many agriculturally important species the fast inhibition of root growth is induced by micromolar concentrations of aluminium ions.

Widely known method of correction of soil pH and overcoming aluminium toxicity problems is surface application of lime. This treatment is not always economically feasible and the slow movement of lime into the deeper sub-surface layers of soil may decrease its efficiency. Thus, the more cost-effective strategy to improve farming on acid soils is the selection and development aluminium tolerant cultivars.

The mechanisms of aluminium tolerance are not fully understood but the large variation between species and also among cultivars within species may suggest some basic systems of tolerance. The differences in response to aluminium ions give the possibility of selection of tolerant forms and also identification and location of Al tolerance genes. Rye is the most Al tolerant species among cereals, the lower tolerance was observed in wheat and barley. Triticale, the synthetic wheat/rye hybrid shows relatively high Al tolerance that is inherited from rye. Generally, the aluminium tolerance of triticale is a little lower than the aluminium tolerance of rye. It is because that some wheat genes control the activation or suppression of rye genes controlling aluminium tolerance (Gustafson and Ross, 1989; Kim *et al.*, 2001). Rye genome seems to have very efficient group of genes of Al tolerance. The Al tolerance genes of rye may be utilised to enhance the tolerance of related cereal species such as wheat and triticale. The study on accumulation of aluminium in root tips of tolerant and sensitive forms of rye suggests the existence two mechanisms of tolerance operating at different external concentrations of aluminium ions. Mechanism of avoidance, preventing apical part of roots from high concentration of aluminium ions, observed in tolerant forms of wheat, seems to be of limited importance in rye. At concentrations of aluminium ions lethal for most accessions of wheat, rye inbred lines exhibit their tolerance or sensitivity despite of similar level of aluminium accumulated in roots (Anioł, 1996). This indicates that in contrast to wheat, the high tolerance in rye is rather connected with the mechanisms of detoxification of aluminium ions already accumulated in the cell.

The use of molecular markers techniques made possible the creation of high-density genetic maps of agriculture plants and localisation of important genes. The identification of markers of Al tolerance in cereals can accelerate the development of cultivars exhibiting enhanced productivity under the condition of Al stress.

The variability of the level of tolerance among accessions of rye gave the possibility to select inbred lines differing in their response to aluminium ions. The use of such plant material created the possibility for identification of markers specific for tolerant and sensitive forms of rye. For identification of these markers we applied PCR system with the use of semi-specific primers targeting intron-exon sequences of plant genes. This system is the modification of the method proposed first by Weining and Langridge (1991), that made its as simple and fast as RAPD but the results obtained are much more informative (Rafalski *et al.*, 1997).

Commonly used method for location of aluminium tolerance genes and their markers on rye chromosomes is the applying a set of wheat-rye addition lines. The testing of tolerance of such sets indicated on presence of tolerance genes on 3R, 4R and 6R rye chromosomes (Anioł and Gustafson, 1984, Anioł, 1995). The testing of aluminium tolerance of wheat/rye addition lines sets (Gustafson and Ross, 1990) and the lines of triticale (Kim *et al.*, 2001) clearly indicates on strong influence of wheat genome on ex-

pression of rye tolerance genes. This makes difficulty in evaluation of influence of particular loci of rye genom on total aluminium tolerance. The identification of markers of aluminium tolerance genes may at least in part overcome these difficulties.

The aim of the study was to confirm the connection of DNA fragments distinguishing tolerant and sensitive forms of rye with the genes affecting aluminium tolerance and to locate them on rye chromosomes using a set of wheat/rye addition lines.

MATERIAL AND METHODS

Winter rye inbred lines differing in response to aluminium ions were selected from breeding program of dr. Madej (Plant Breeding and Acclimatisation Institute - Radzików). Under the condition of laboratory test, inbred lines designated as L1, L2 and L3 exhibited sensitivity to 0.5 mM concentration of aluminium ions while inbreds L16, L17 and L18 revealed high level of tolerance. F_2 segregants were obtained from crosses L1 × L16, L2 × L17 and L3 × L18.

A set of wheat-rye addition lines (Chinese Spring/Blanco) obtained from prof. Łukaszewski (University Riverside, CA) was used for chromosomal location of markers of tolerance.

The DNA of inbred lines and F2 segregants of rye was isolated from bulks containing equal quantities of the leaf tissue of 15-25 plants. In the case of wheat-rye addition lines the DNA was isolated from single plants in which the presence of alien chromosomes was verified using cytological and molecular methods (Wiśniewska and Rafalski, 2003). The DNA was prepared according to the procedure of Davis *et al.* (1986). The quantity of DNA was evaluated fluorometrically according to the procedure described in the TKO fluorometer booklet (Hoefer Sci., San Francisco, USA).

The polymerase chain reactions were carried out using a Uno II thermocycler (Biometra, Gottingen, Germany). The 20 μl reaction mixture contained: 15 ng of genomic DNA template, 1,0-1,2 μM of primer, 200 μM each of dATP, dCTP, dGTP and dTTP, 2 mM $MgCl_2$, 1,0 unit of Taq polymerase (MBI, Fermentas, Vilnius, Lithuania) and an appropriate reaction buffer. Amplification was carried out in two steps: in the first seven cycles the annealing temperature was 2°C higher and during the following 33 cycles 6°C higher then the temperature calculated from primer length and composition. In all the cycles, the denaturation was carried out for 40 sec. at 95°C, annealing for 1 min. and amplification for 2 min. at 72°C. The amplification products were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide and photographed under UV light.

The DNA fragments differentiating tolerant and sensitive forms of rye were cut out from agarose gel and placed into 0,5 ml Eppendorf tubes with small holes stopped with siliconized glass fibre. The tubes were placed into 1,5 ml Eppendorf tubes and centrifuged (10 min., 12 000 r.p.m.). After centrifugation the agarose gel was denatured 2x by dipping in liquid nitrogen, washed with 40 μl TE buffer and centrifuged. The DNA was precipitated using appropriate volume of ethyl alcohol, centrifuged, washed with a small volume of 80% alcohol and after drying diluted with 40-50 μl of TE buffer For reamplification. 4-5 μl of DNA solution was used as a matrix. The remaining components of reaction mixture and also denaturation and amplification temperatures were as described above. Reamplification was carried out during 33 cycles with annealing tem-

perature 6°C higher then the temperature calculated from primer length and composition. The samples of reamplified DNA fragments were separated on agarose gel and their quantity were evaluated by comparison with DNA size standard. The size of DNA fragments were determined using "Fragment NT" (Molecular Dynamics, Sunnyvale, CA,USA) software.

The DNA fragments differentiating tolerant and sensitive forms of rye were labelled with digoxygenin-dUTP using random primed system or PCR method according to Saiki *et al.* (1985). During labelling using random primer system it was applied the procedure described in the booklet for DIG DNA labelling and detection kit (Roche, Mannheim, Germany). The 20 µl reaction mixture contained 80 - 150 ng of DNA. The incubation time was 16-18 hours at 37°C.

The reaction mixture for labelling using PCR method contained 15-30 ng of DNA and typical components of PCR reaction in 20µl volume. The deoxynukleotide mix was supplemented with dNTP labelling mixture containing 35 µM DIG-dUTP. Labelling was carried out during 33 cycles as for reamplification DNA fragments. Digoxygenin labelled DNA fragments were precipitated with ethanol, centrifuged and after drying diluted in 40-50 µl of TE buffer.

Southern blotting of PCR amplified DNA fragments from agarose gel onto nylon membranes was performed according to procedure described in "Molecular Cloning" (Maniatis *et al.* 1982).

Hybridisation, immunological detection and colour development were carried out according to procedure proposed by producer DIG-DNA labelling and detection kit (Roche, Mannheim, Germany). The only modification of procedure was that the concentration of commercial blocking reagent was decreased to 0.5% and hybridisation buffer was supplemented with sonicated DNA of rye (Dańkowskie Złote) at concentration 5 µg/ml. Prehybridisation temperature was 60°C. After 3 hours of prehybridisation DIG-labelled probe was added (5-10 ng/ml). The incubation with hybridisation buffer was 16 hours at 68°C. During post-hybridisation and stringency washes the procedure described by Metzlaff *et al.*(1986) was followed.

RESULTS AND DISCUSSION

Although rye indicates high tolerance to aluminium ions, there exists essential variability of this tolerance between rye genotypes. The laboratory screening of winter rye gave possibility to select inbred lines, which exhibited the contrasting and quite uniform response to aluminium ions. Under the condition of laboratory test (Anioł, 1984), the regrowth of root tips of sensitive inbreds was fully repressed by 1mM concentration of Al ions, while the regrowth in the tolerant once was only slightly affected. From three crosses between sensitive and tolerant lines we selected F_2 segregants, which indicated the response to aluminium ions similar to response of parental lines. For identification markers of aluminium tolerance we applied a bulked segregant analysis method of Michelmore *et al.* (1991). According to this method the markers linked to the loci determining the trait of interest can be identified by comparing two pooled DNA samples of individuals from a segregating population. This population originates from single cross between two forms with contrasting response to parameter studied. In rye, such simple comparison of PCR amplification products of pooled DNA samples of

F_2 segregants differing in response to aluminium ions appeared to be insufficient. The comparison of banding patterns of tolerant and sensitive segregants revealed polymorphic bands of different size. The high frequency of polymorphic bands suggested that considerable part of its seems to be false positives and polymorphic fragments are unlinked to the character studied (Fig.1). To increase the probability of proper markers selection we compared the amplification products of three tolerant and three sensitive F_2 segregants and their parental inbred lines. Among about 1600 bands amplified using 84 semi-specific primers 40 DNA fragments were selected. These fragments differentiated at least two pairs of inbreds and segregants and indicated similar size. About half of primers used revealed polymorphisms between tolerant and sensitive forms of rye. The banding patterns generated by some primers especially primers 18 bases in length were very complex and polymorphic. This complexity causes some difficulties with identification of DNA fragments differentiating sensitive and tolerant genotypes. Sufficiently clear banding patterns of amplified DNA fragments were obtained using shorter primers as for example ET28/12 (Fig.2). The DNA fragment 990 bases in length characterising all tolerant forms amplified using this primer was easy identified and separated from the gel. After reamplification the amount of DNA varied from 200 to 800 ng and was sufficient for preparation digoxygenin labelled molecular probes and their use in further experiments.

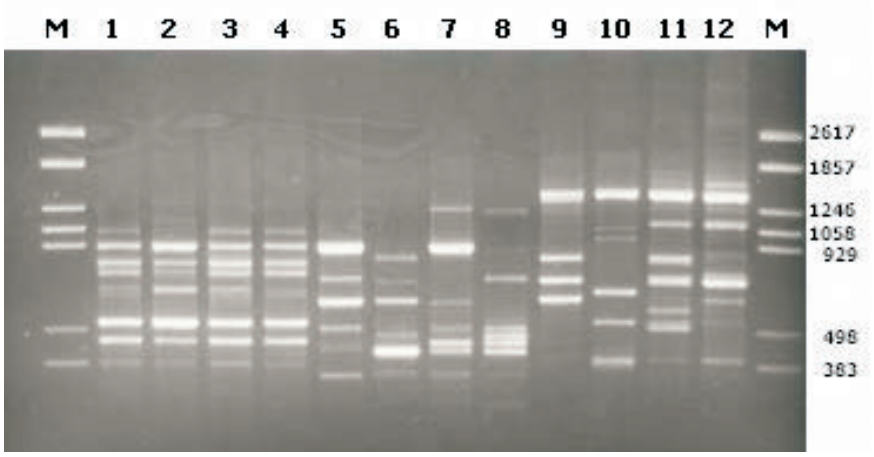

Fig.1. Amplification of DNA of sensitive and tolerant segregants F_2 using primers IT43/10 (lanes 1-4), IT44/10 (lanes 5-8) and IT45/10 (lanes 9-12).
Lanes 1, 3, 5, 7, 9, 11 - sensitive F_2 segregants
Lanes 2, 4, 6, 8, 10, 12 - tolerant F_2 segregants
M - DNA size marker (bp)

Because of the complexity of banding patterns the evaluation of chromosomal location was not possible by direct comparison of the amplification products of tolerant and sensitive forms of rye and the set of wheat/rye addition lines. To assign the DNA fragments to particular chromosome they were used as molecular probes in hybridisation with genomic DNA (dot blots) and with amplified products (Southern blots). For most of DNA fragments their chromosomal location using of dot blot technique gave not satisfactory results. Despite of accurate selection of single plants representing wheat lines with confirmed additions of rye chromosomes, the hybridisation signal appeared

in all dots and only quantitative differences suggested particular location of some fragments (Wiśniewska and Rafalski, 2003). Similar difficulties with the use of dot blot technique for chromosomal location of RAPD markers were described by Gallego *et al.* (1998).

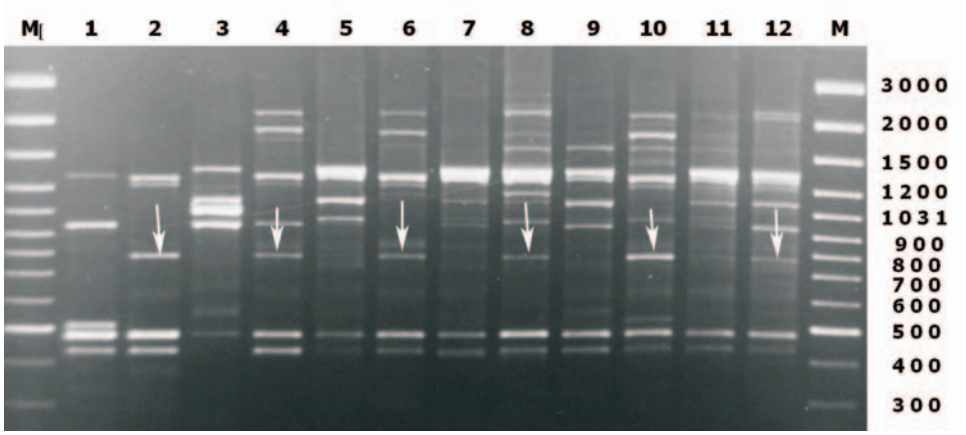

Fig. 2. Amplification of DNA fragments of aluminium sensitive and tolerant rye inbred lines and their F_2 segregants using ET22/12 primer.
Lines 1, 5, 9 – aluminium sensitive lines (L1, L2, L3)
Lines 2, 6, 10 – aluminium tolerant lines (L16, L17, L18)
Lines 3, 7, 11 – F_2 aluminium sensitive segregants
Lines 4, 8, 12 – F_2 aluminium tolerant segregants
M – DNA size standard (bp)

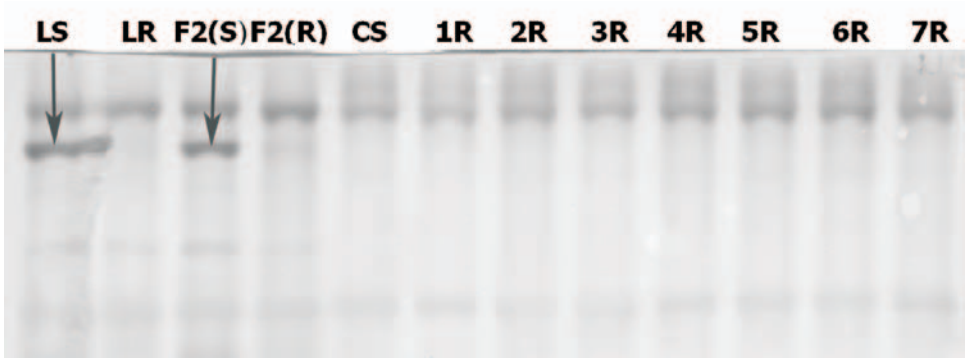

Fig.3. Hybridization of probe created from 920 bp DNA fragment characterising sensitive forms of rye to DNA of sensitive (LS) and tolerance (LR) inbred lines of rye, their sensitive [F2(S)] and tolerant [F2(R)] segregants F_2 to DNA of wheat/rye (Chinese Spring/Blanco) addition lines amplified with PCR method using ET26/12 primer

The more satisfactory results were obtained using Southern hybridisation to amplified DNA of sensitive and tolerant forms of rye and the set of wheat-rye addition lines. The application of two method of labelling results the number of probes exceeded the number of DNA fragments differentiating tolerant and sensitive forms of rye. Results of hybridisations revealed that both methods gave similar results. Among 40 fragments

tested, the hybridisation patterns of probes created from 12 DNA fragments confirmed their connection with character studied and in the case of probes created from eight DNA fragments was also designated their chromosomal location. The probes prepared by labelling of four DNA fragments showed evident connection with tolerance or sensitivity, but not hybridised to any addition line. The example of such hybridisation pattern is presented on Fig.3. The probes prepared from 920 bp DNA fragment characterising sensitive form of rye have not indicated any hybridisation signal with the DNA of addition lines. The lack of hybridisation signal results probably from the response of rye variety Blanco to Al ions. This variety is moderately aluminium tolerant and absence of dominant markers of aluminium sensitivity seems to be reasonable. This situation could confirm the results of hybridisation of probe prepared from 670 bp DNA fragment isolated from sensitive forms of rye. This DNA fragment have not hybridised to any addition lines. The codominant nature of the fragment revealed by presence of bit smaller DNA fragments in tolerant forms suggested its chromosomal location. To DNA of 4R chromosome hybridised evidently only the marker of tolerance (Fig.4.). The lack of hybridisation signal to wheat/rye addition lines in the case of three other probes results probably also from characters of Blanco variety and its genetic distance from rye inbred lines of Polish origin. These probes could be tested using another set of addition lines. Despite of these difficulties most of probes characterising tolerant forms of rye hybridised to DNA of particular addition line. Three probes hybridised to DNA of chromosome 4R. The example of such hybridisation pattern is presented on Fig.5. The probes created from two DNA fragments hybridised strongly to DNA of 6R addition line (Fig.6). Single probes hybridised to chromosomes 1R, 2R and 3R. In some other cases probes hybridised weakly to another DNA fragments than they originated because of existence of unknown common sequences. This can also explain ambiguous results of dot-blot hybridisation.

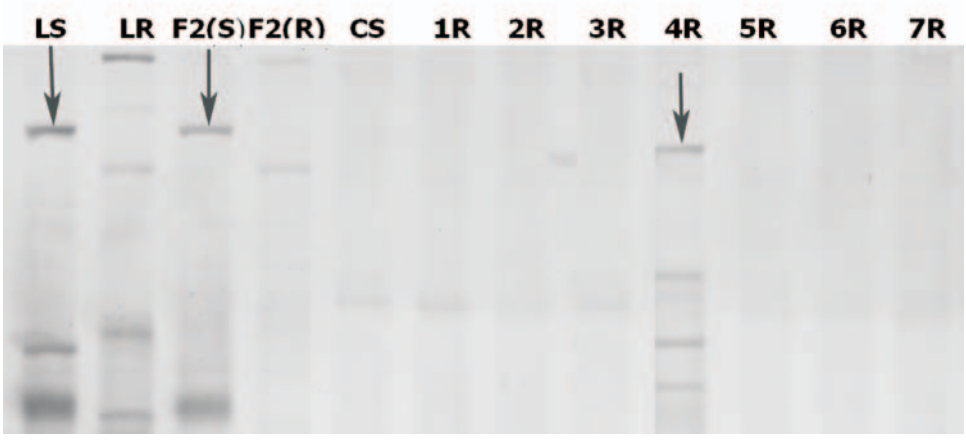

Fig.4. Hybridization of probe created from 672 bp DNA fragment characterising sensitive forms of rye to DNA of sensitive (LS) and tolerance (LR) inbred lines of rye, their sensitive [F2(S)] and tolerant [F2(R)] segregants F_2 to DNA of wheat/rye (Chinese Spring/Blanco) addition lines amplified using ET3/18 primer

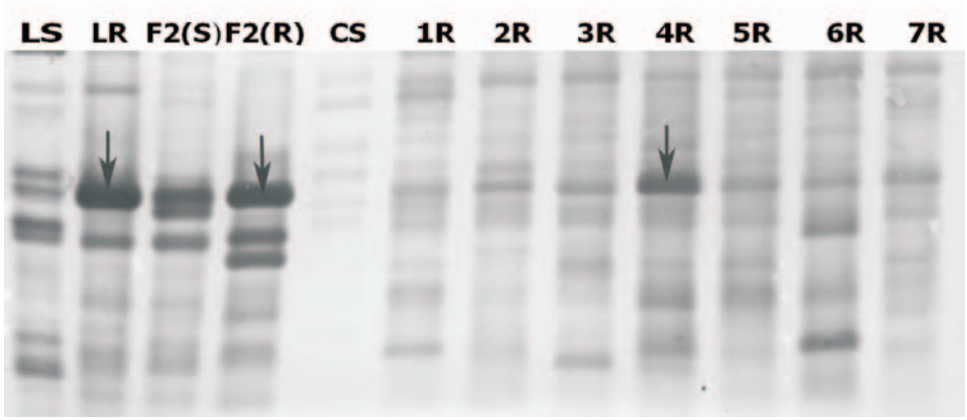

Fig.5. Hybridization of probe created from 560 bp DNA fragment characteristic for tolerance forms of rye to DNA of sensitive (LS) and tolerance (LR) inbred lines of rye, their sensitive [F2(S)] and tolerant [F2(R)] segregants F_2 to DNA of wheat/rye (Chinese Spring/Blanco) addition lines amplified with PCR method using ET12/18 primer

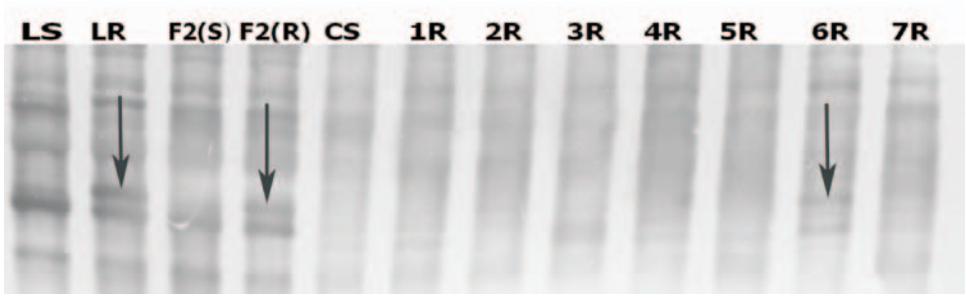

Fig.6. Hybridization of probe created from DNA fragment of 689 bp characteristic for tolerant form of rye to DNA of sensitive (LS) and tolerance (LR) inbred lines of rye, their sensitive [F2(S)] and tolerant [F2(R)] segregants F_2 to DNA of wheat/rye (Chinese Spring/Blanco) addition lines amplified with PCR method using IT45/10 primer

CONCLUSIONS

PCR method with the use semi-specific primers appeared to be useful for identification of aluminium tolerance markers in rye.

For chromosomal location of markers the dot-blot hybridisation system appeared to have limited use. The quantitative differences of hybridisation signals observed can be treated as preliminary results.

The results of Southern hybridisation to DNA of PCR amplified fragments confirmed relationships of isolated DNA fragments with tolerance or sensitivity to Al ions and their location on particular chromosomes of rye.

Hybridisation patterns of eight DNA fragments enabled their location on specific chromosomes of rye. Three DNA fragments were located on chromosome 4R, two on chromosome 6R and single DNA fragments on chromosomes 1R, 2R and 3R.

The lack of hybridization signal of some fragments to DNA of addition lines Chinese Spring/Blanco results probably from genetic distance between Polish accessions studied and moderately tolerant rye Blanco.

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