Molecular analysis of wheat dihaploid lines in scope of selection for common bunt resistance

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Abstract  Common bunt of wheat, caused by two heterobasidiomycete fungi of genus *Tilletia*, *Tilletia laevis* and *Tilletia tritici*, produces significant damages to wheat especially when chemically untreated seeds are sown. The identification of molecular markers linked to common bunt resistance can improve phenotypic selection of resistant lines as the trait is expressed late and is highly influenced by environment. The objective of this study was to analyse the polymorphism at the molecular level between wheat dihaploid lines (DHLs) (resistant to bunt), selected from two crosses (GP384=99419G4-1A/1-1/98047G14-21INC1 and GP369=99419G4-1A/1-1/00356G8-1) and eight susceptible lines and cultivars (from 15 to 22), in order to find markers to be used for selection of resistant lines. RAPD (Random Amplified Polymorphic DNA) technique by testing a total number of 38 decamer primers has been used. We have also used a pair of specific PCR primers FSD (forward) and RSA (reverse), (Laroche et al., 2000) for Bt-10 bunt resistance gene. Among the random primers tested, primers OPA16, Mic14, OPC10, Mic03, UBC570 and Mic07 revealed polymorphic fragments between resistant and susceptible lines to bunt. Thus, primers Mic14 and OPA16 have generated polymorphic DNA fragments of 630 bp and 900 bp respectively. These were present at almost all the dihaploid lines (resistant) but not at the susceptible wheat lines and cultivars. Specific PCR primers FSD and RSA generated a polymorphic fragment of 1450 bp present at almost all the dihaploid lines (resistant) but not at the susceptible lines and cultivars. These results suggest that polymorphic DNA fragment of 1450 bp from specific primers FSD and RSA might be considered as a marker for some of the common bunt resistance genes. To increase the reliability and the reproducibility of the identified RAPD fragments as polymorphic markers further genetic analyses are necessary to convert them into SCAR markers (Sequence Characterized Amplified Region).

Key words  RAPD primers, polymorphism, wheat, dihaploid lines, common bunt

Common bunt of wheat is an infectious disease distributed in all over the wheat-growing regions that produces significant damages of grain yield and reduces the quality of seeds at the infected plants. The disease is caused by two species of the genus *Tilletia*, *Tilletia laevis* Kühn and *Tilletia tritici* (Bjerk.) Wint., and can be found both on common wheat and on the durum wheat [9]. It is spread by spores which persist on the seeds or in the soil and they develop during the seed germination. The symptoms are visible only when the plants have reached maturity; the kernels of the infected plants are replaced by the toxic fungus spores emanating a specific smell of trimethylamine [9]. The yield losses can reach up to 40 % from yield value when the seeds are untreated or the favorable conditions for disease development occur. [4]. The losses affect the grain quality because in the infected heads the kernels are replaced by toxic spores making them unsuitable for making bread or feeding animals. Since 1950s, the disease has been controlled by seed treatments with chemicals, a very efficient way of control [4]. However, it is necessary to search for new sources of resistance to common bunt that can be used in breeding for resistance. In organic farming using resistant cultivars is the single way of disease control because the seed treatments are not allowed. [5].

Current commercial cultivars are less resistant to common bunt, few are moderate resistant or resistant [2]. However, sources of resistance are available in the related species and in introgressions lines from related

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species (*Triticum boeoticum*, *T. dicoccoides*, and *Aegilops*, *Triticum monococcum*, *Triticale*) [6]. The resistance to common bunt in wheat is controlled by 15 major resistance genes (*Bt*) (specific resistance); to date a number of 15 resistance genes have been identified in wheat [9].

Dihaploid lines (DHLs) are completely homozygous and they contain two identically set of chromosomes, having the advantage that do not segregate.

The Random Amplified Polymorphic DNA (RAPD) technique is based on polymerase chain reaction (PCR) using a single primer with short (10 nucleotide), arbitrary sequence [10]. The polymorphisms are revealed as the presence or the absence of the band at the individuals, the markers being dominant. The amplification products (0,5-5 kb) are separated by electrophoresis in agarose gel.

The objective of this study was to identify the molecular polymorphism between wheat dihaploid lines being homozygous and they contain two identically set of chromosomes, having the advantage that do not segregate.

Material and Methods

Plant material
For genetic analysis we have used four wheat dihaploid lines (DHLs) selected from two crosses:
- 28 lines selected from GP369 cross having the following genealogy GP369= 99419G4-1A/1 1/00356G8-1,
- 41 lines selected from GP 384 cross with the genealogy GP384= 99419G4-1A/1-1/98047G14-21NC1. We also used, as susceptibles forms the following lines and cultivars: 15-FARMEC, 16-DELABRAD, 17- F96869G1-108, 18-GLOSA, 19-BOEMA, 20-JIANA, 21- CRINA, 22-DROPPIA.

Selection of dihaploid lines used has been done based on sources for resistance to common bunt (*Tilletia spp*.) present in their genealogy. Parental form 99419G4 (designed 23), is a line with high resistance to common bunt in the field. In our analysis a polymorphic band of 630 pb has been amplified at this line with the primer Mic14 absent at all the susceptible lines. The susceptible lines and cultivars were tested for resistance and they showed susceptibility to common bunt in the repeated tests conducted in the field.

RAPD primers used
In the RAPD analysis, a number of thirty eight primers were tested on the dihaploid lines and the susceptible lines and cultivars, to detect polymorphism. Some of them, did not amplify any PCR products while others amplified non-polymorphic bands. Six of the primers used have amplified polymorphic bands between wheat resistant dihaploid lines and susceptible lines and cultivars, (Table 1).

FSD and RSA specific primers
These primers were designed based on polymorphic fragment of 590 bp produced by RAPD technique. They have been tested by Laroché an his coworkers [3], permitting rapid identification of 275 bp marker linked to *Bt*-10 resistance gene at 22 resistant cultivars and individual lines. The primers have the following nucleotideic sequence: FSD-forward (5'-GTTTATCTTTTATTTTC- 3') and RSA-reverse (5'-CTCCTCCCCCA- 3') and annealing temperature (°C) at 42°C.

For the RAPD primers the DNA amplification was performed using the following thermal cycles: an initial cycle of 3 min (94°C); 45 cycles of 1 min denaturation (95°C), 1 min annealing (34°C, depending on optimum annealing temperature of the primers), 2 min elongation (72°C); and a final extension step of 7 min (72°C). The PCR products were separated by electrophoresis in 1.4% agarose gels in Tris–borate–EDTA buffer and than were visualized by staining with ethidium bromide (EtBr). The gels were photographed under UV light and the images were stored using Alpha Innotech machine.

DNA extraction
DNA was extracted using cetethyltrimethyl-ammonium bromide (CTAB) method [8]. The DNA concentrations were quantified by use of a spectrophotometer.

Polymerase chain reaction (PCR)
To amplify the DNA, PCR reactions were performed using a reaction mixture of 25 µl containing: 5x PCR Green GoTaq Flexi buffer, 1,5mM MgCl2, 2µM primer decamer, 200 µM of each dNTP, 0.5 units of *Taq* DNA polymerase and 20 ng of genomic DNA. For PCR specific primers FSD and RSA amplifications were carried out in the same conditions as for random primers, except the number of the thermal cycles and the annealing temperature of the primers. To reduce the number of nonspecific amplification products the annealing temperature was optimized at 42°C.
Results and Discussions

Analysis using FSD and RSA specific primers

Primers FSD (forward) and RSA (reverse) usually amplify a DNA fragment of 275 bp as specific marker for *Bt-10* resistance gene. We have tested these primers at wheat dihaploid lines we used and they produced a polymorphic DNA fragment of 1450 bp. This was present in almost all the dihaploid lines from both crosses but not in the susceptible forms, (Fig. 1). The primers also showed another polymorphic band of approximately 1000 bp and some of the bands were identical in both the resistant and susceptible lines. Since the band of 1450 pb was amplified at all dihaploid lines from both crosses (with resistance to bunt), in cross GP 384, except lines 4.26 and 4.28 but not at the susceptible forms this suggests that the fragment of 1450 bp might be a putative markers for one of the resistance genes.

RAPD analysis

Primers were initially tested on the several dihaploid lines (DHLs) from each cross and on the susceptible forms (Fig. 2). Then the polymorphic primers were tested in all dihaploid lines. Primers OPA16, OPA17, OPA18, Mic07, OPC10, Mic14 generated polymorphic DNA fragments which could discriminate the wheat dihaploid lines (resistant) from line 17 (susceptible). (Fig. 2).

Out of these, primers OPA16 and Mic14 revealed polymorphic fragments in dihaploids lines (resistant) which were absent in the susceptible wheat lines and cultivars. Thus, primer Mic14 revealed a polymorphic fragment of 630 bp present in almost all the dihaploid lines from both crosses but absent in the susceptible lines and cultivars, (Fig. 3). Primer OPA16 also generated a polymorphic fragment of approximately 900 bp between dihaploid lines (resistant) and the susceptible forms. Almost all the dihaploid lines, from both crosses, showed this fragment but none the susceptible forms (17-F96869G1-108 and 21-CRINA), (Fig. 4 and 5).

![Fig. 1. PCR products from FSD/RSA specific primers. The fragment of 1450 bp have been amplified at dihaploid lines from cross GP 384 (4.1-4.41) but not at the susceptible forms (17 and 21). M-100 bp molecular ladder.](image-url)
Fig. 2. Test of primers OPA17 (A), OPA18 (B), Mic07 (C), OPC10 (D) on dihaploid lines from cross GP369 (9.1-9.6) and susceptible line (17-F96869G1-108), M-100 bp molecular ladder.

Fig. 3. RAPD amplification products obtained with primer Mic14 in dihaploid lines from cross GP 369 (resistant to bunt) and in the susceptible forms 17-F96869G1-108 and 21-CRINA, L-100 bp molecular ladder.

Fig. 4. RAPD amplification products obtained with primer OPA16 in dihaploid lines from cross GP 369 (resistant to bunt) and in the susceptible forms 17-F96869G1-108 and 21-CRINA, L-100 bp molecular ladder.
Fig. 5. RAPD amplification products obtained with primer OPA16 in dihaploid lines from cross GP 348, 4.29-4.41 (resistant to bunt) and in the susceptible forms 17- F96869G1-108 and 21- CRINA, L-100 bp molecular ladder.

Conclusions

1. The specific primers FSD and RSA for Bt-10 gene for resistance to common bunt, have amplified in dihaploid lines used a polymorphic fragment of 1450 bp different of the fragment of 275 bp reported by Laroche, 2000. The presence of this polymorphic fragment as a marker in the wheat dihaploid lines tested suggests the presence of resistance genes in almost all the wheat dihaploid lines from both crosses.

2. In RAPD analysis some primers gave a good amplification at all tested wheat lines. The primers that revealed reproductible DNA polymorphic fragments were Mic 14 and OPA 16. They amplified a band of 600 bp and 900 bp respectively. Their presence at dihaploid lines from both crosses and absence at the susceptibles lines and cultivars suggest that these fragments are associated with some of the resistance genes to common bunt.

3. To increase the reliability and the reproductibility of the identified RAPD polymorphic fragments and to establish a putative association between these fragments and common bunt resistance genes, further work is necessary to convert them into specific markers by Sequence Characterized Amplified Regions (SCAR) technique.

References