Evaluation of spring barley genotypes with different susceptibility to Fusarium head blight using molecular markers

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ABSTRACT

One of cereal diseases that has gained a greater importance for growers, processing industry as well as for breeders in many regions of the world is Fusarium head blight (FHB) caused by fungi of the *Fusarium* genus. The objectives of this study were to test diversity among spring barley breeding lines exhibiting various sensitivity to FHB and to find RAPD markers and AFLP markers that will distinguish between susceptible and resistant and/or moderately resistant genotypes. A test of a set of spring barley genotypes artificially infected by fusaria in field trials was carried out. Based on the results from field and laboratory evaluation and deoxynivalenol (DON) content assessment, barley genotypes with different responses to FHB were selected. The genotypes were hybridized and doubled haploid (DH) lines were derived in F_1 generation using the *in vitro* androgenesis method. Initial parental components and derived DH lines were tested for FHB infection and DON content. A set of parental genotypes of spring barley was tested with 80 RAPD markers. A RAPD marker (H30) was detected which enabled to distinguish between very susceptible parental genotypes and other resistant or moderately resistant spring barley genotypes based on the fragment of about 1300 bp. This specific product was screened in 23 DH lines derived from crosses of parental genotypes of spring barley and detected in 10 DH lines. During the study, some DH lines were selected that exhibited improved resistance to *Fusarium* infection. A low infection level and low DON content was found in the line DH 4/2 derived from CI 4196 × Foster. The AFLP technique was used to analyse parental genotypes of spring barley. The detected markers can be further evaluated and employed to select breeding materials.

Keywords: spring barley; Fusarium head blight; deoxynivalenol; doubled haploid lines; RAPD; AFLP

Fusarium head blight (FHB) is a worldwide distributed cereal disease that is caused by fungi of the *Fusarium* genus. Infection of plants can result in yield reduction and accumulation of toxic secondary fungal metabolites, mycotoxins in kernels (Windels 2000, Takeda 2004). The number of papers dealing with FHB on barley is generally much lower than that of research studies on wheat. It is due to a much greater economic importance of wheat as a cereal crop, but as well as to the fact that FHB causes larger economic losses in wheat than in barley. Though the two crops are attacked by identical FHB pathogens, there are differences in their infection responses. In general, barley is less susceptible to FHB than wheat. Grain yield is affected by FHB more in wheat than in barley, however a higher mycotoxin concentration is observed in the latter due to outer cover layers (lemma and palea) that are parts of the kernel from harvest to end use (Steffenson 2003). Produced toxins are harmful to human health and can induce serious diseases. The mycotoxins are deactivated neither in malting nor in brewing processes. Moreover, they can influence beer flavour and cause gush-

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ing. If barley is fed to animals, deoxynivalenol (DON), which is a main inhibitor of protein synthesis, induces growth retardation and it weakens organism defence functions, decreases appetite to intake feeds and conditions neural defects associated with aggressiveness and cannibalism. Food contamination by fusaria mycotoxins is thus considered as a serious agricultural problem that markedly affects economics, international trade, human and animal health (Schwarz 2003). The best way to avoid FHB is to employ integrated crop management practices, apply fungicides and introduce new more resistant cultivars. However, breeding new resistant genotypes is not easy since FHB resistance is a complex character, genetically determined by loci with relatively low effects and variable level of environmental effects (Kolb et al. 2001). One of the possibilities to identify genotypes resistant to FHB, and thus to promote development of new more resistant genotypes, is to use molecular markers in breeding processes (Mueller and Wolfenbarger 1999, Armstrong et al. 2001). The objectives of the study were to test diversity among barley breeding lines exhibiting various sensitivity to FHB and to detect RAPD (Random Amplified Polymorphic DNA) and AFLP (Amplified Fragment Length Polymorphism) markers that enable to distinguish between susceptible and resistant and/or moderately resistant genotypes.

MATERIAL AND METHODS

Plant material. Seven spring barley initial genotypes with declared resistance or susceptibility to FHB (Table 1) and 23 DH lines (Table 2) were tested in 2005. DH lines were developed using the *in vitro* androgenesis method after hybridization among initial parents according to Vagera and Ohnoutková (1993).

Evaluation of resistance to FHB

Field trials. The trials were carried out at the location of the Agricultural Research Institute Kroměříž, Ltd. Plants were cultivated using standard agronomic practices in two replications at 1 m^2 plots. The plots were artificially inoculated with the spores of *Fusarium culmorum*, FC-417/02 isolate. The concentration of the inoculum was adjusted to 6 million conidia per ml. Inoculation was carried out at full anthesis (DC 65) in terms depending on genotypic differences. The plants were visually evaluated 21 days after inoculation using the scale by Horsfall-Barrett as a percentage of dry spikelets due to disease infection.

Laboratory test (paper rolls). Hundred grains in two replications were tested per each accession as described previously by Tvarůžek et al. (2003). The percentages of germinating and infected grains were checked.

Evaluation of DON content. The DON content was determined by the quantitative enzyme-linked immunosorbent assay (ELISA) using the kits RIDASCREEN FAST DON (R-Biopharm GmbH, Darmstadt, Germany) that are approved by the Association of Official Analytical Chemists (AOAC). The guidelines of the manufacturer were followed for sample preparation, extraction and assessment. Measurements were performed on spectrophotometer MRX II (Dynex, USA) and data were analyzed using the Revelation software (Dynex, USA).

Methods for RAPD analyses. Genomic DNAs were extracted from leaves of seedlings (6 days old plants) using DNAesy Plant Mini Kit (Qiagen Firm). The reaction mix consisted of a total volume for reaction 25 µl, 1× buffer for *Taq* polymerase, 0.4 U *Taq* polymerase (Finnzymes), 0.25mM mixture dNTP, 30 ng primer and 30 ng DNA. Reaction conditions: 1 min initial denaturation at 94°C; 45 cycles – 1 min

Table 1. Characteristics of the spring barley cultivars and lines and their responses to Fusarium head blight

Genotype	Pedigree	Origin	Row type	Response to FHB
Chevron	CIho 1111 (PI 38061) = Landrace from Luzerne	CHE	6	resistant
PEC 210	Realised in Brazil as Embrapa 128	BRA	2	resistant
CI 4196	PI 64275 (Hang wang ta mai) = Landrace from Beijing	USA	2	resistant
Zao Zhou 3	Cultivar in East China, Zhejiang University, Hangzhou	CHN	2	moderately resistant
6NDRFG-1	PI 615583; North Dakota Agric. Experiment Station, USA	USA	6	moderately resistant
Foster	Robust/3/Hazen//Glenn/Karl	USA	6	very susceptible
PI 383933	Kanto Nijo 2 = Ko. 1018/Kyoto Nakate from Japan	USA	6	very susceptible

denaturation at 94°C, 2 min annealing at 35°C, 1 min elongation at 72°C; followed by final synthesis at 72°C for 10 min. Electrophoretic separation was carried out on 1.5% agarose gel and visualization using ethidium bromide.

Methods for AFLP analyses. Genomic DNAs were extracted from leaves of 14 days old plants by a method based on selective precipitation in CTAB (cetyltrimethylammonium bromide) (Saghai-Maroof et al. 1984). Quality and quantity were estimated using electrophoresis and spectrophotometry. The DNA concentration was determined by comparison with the weight standard lambda/*Hind*III. Furthermore, 0.5 µg DNA at the volume of 5.5 µl was used. Restriction segregation using enzymes *Eco* RI and *Mse* I, ligation adapters and pre-selective amplifications were performed according to the Manual of Perkin Elmer Firm (Rev. Manual 1995, Part No. 569933) except for *Taq* polymerase. *Taq* polymerase of the Qiagen Firm (1 U reaction, relevant buffer, 5mM dNTP, 10µM selective primer – *Mse*I and 1µM *Eco*RI labelled primer) was used for selective amplification. The thermocycler Perkin-Elmer 9600 at the profile recommended in the AFLP manual was used. Amplification products were separated by capillary electrophoresis using Perkin-Elmer Genetic Analyser ABI 310. The results were evaluated by GeneScan and Genotyper software.

Data analysis. The electrophoreograms were converted into binary matrices reflecting the presence (1) or absence (0) of resulting alleles. The matrices were assessed by FreeTree software, ver. 9.1 using the UPGMA construction method and similarity coefficient according to Nei and Li (1979). TreeView software, ver. 1.6 was used for graphical expression of the matrix.

RESULTS AND DISCUSSION

Seven spring barley cultivars were tested in field trials. Based on the results, visual evaluation in the field, laboratory tests of fusaria occurrence in paper rolls and DON content in grain, it was confirmed that a sensitivity level of these genotypes to FHB corresponded with the declared resistance/susceptibility under climatic conditions of the Czech Republic. In our trials, however, none of the tested genotypes with declared resistance was bellow the maximum limit for DON content, which is 1.25 ppm in the EU countries since July 1, 2006. In the line CI 4196, which is considered as the most resistant in the field among two-rowed barleys, ear infection corresponded with fusaria occurrence in grain on a level of 10% and DON content of 2.04 ppm. In the cultivar Chevron, which is considered to be the most resistant in the field among six-rowed barleys, the ear infection was 30%, fusaria occurrence in grain 9% and DON content 2.28 ppm. In our trials, an insignificant correlation (r = 0.19) was calculated between the percentage of ear infection and fusaria percentage in the laboratory test. Similarly, insignificant correlations were found between ear infection percentage and DON content (r = 0.31) and between fusaria percentage in the laboratory test and DON content (r = 0.36). By contrast, other authors (Buerstmayr et al. 2004, Špunarová et al. 2005) reported high correlations between the studied characteristics. However, Schaafsma et al. (2004) stated that a direct correlation between scabby grains and mycotoxin content might not exist because not all *Fusarium* species are toxigenous. For instance, *Microdochium nivale* var. *majus* and *M. nivale* var. *nivale,* which are very frequent in

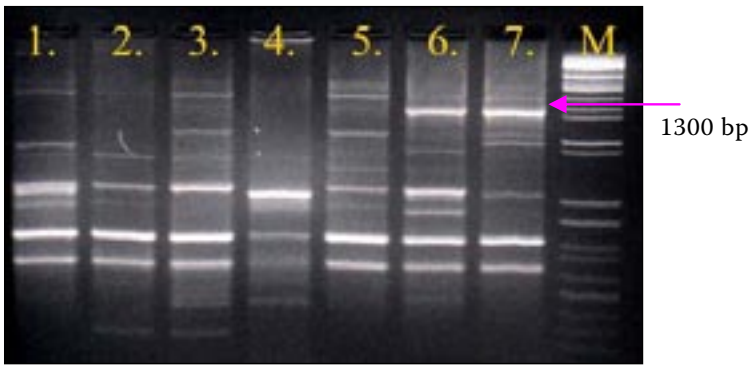

Figure 1. Electrophoreogram of 7 spring barley parental genotypes after RAPD analysis using the marker H30; (1) Chevron, (2) PEC 210, (3) CI 4196, (4) Zao Zhou 3, (5) 6NDRFG-1, (6) Foster, (7) PI 383933; the arrow indicates the product of 1300-bp size characteristic for a group of susceptible genotypes

Table 2. Evaluation of spring barley doubled haploid lines in comparison to check varieties

Cultivar/line	Origin/Pedigree	Field evaluation (%)	Laboratory test (%)	DON content (ppm)	Presence of specific product
Chevron	CHE	30	9	2.28	0
PEC 210	BRA	5	12	2.41	0
CI 4196	USA	10	10	2.04	0
6NDRFG-1	USA	5	15	9.93	0
Zao Zhou 3	CHN	7	18	7.93	0
Foster	USA	8	29	14.94	1
PI 383933	USA	10	44	12.37	1
DH 1/1	Chevron × Foster	66	59	12.89	1
DH 2/2	Chevron × PI 383933	51	95	12.21	0
DH 2/3	Chevron × PI 383933	49	100	11.87	0
DH 3/1	PEC 210 × Foster	40	69	10.47	0
DH 3/3	PEC 210 × Foster	26	95	8.55	0
DH 3/4	PEC 210 × Foster	50	98	9.75	0
DH 3/6	PEC 210 × Foster	44	47	6.88	0
DH 3/9	PEC 210 × Foster	17	99	9.02	0
DH 3/10	PEC 210 × Foster	66	57	3.90	0
DH 4/2	CI 4196 × Foster	13	60	3.30	0
DH 4/3	CI 4196 × Foster	45	59	16.04	1
DH 5/1	CI 4196 × PI 383933	56	34	7.18	1
DH 5/3	CI 4196 × PI 383933	27	62	8.03	1
DH 5/12	CI 4196 × PI 383933	75	36	17.34	1
DH 5/14	CI 4196 × PI 383933	70	49	10.55	1
DH 5/15	CI 4196 × PI 383933	34	72	12.44	0
DH 5/16	CI 4196 × PI 383933	54	65	15.53	1
DH 5/17	CI 4196 × PI 383933	79	77	4.62	1
DH 7/1	6NDRFG-1 × PI 383933	78	43	30.00	1
DH 9/2	Zao Zhou 3 × PI 383933	40	98	14.42	1
DH 9/4	Zao Zhou 3 × PI 383933	49	99	12.34	0
DH 9/6	Zao Zhou 3 × PI 383933	33	98	17.32	0
DH 9/10	Zao Zhou 3 × PI 383933	84	95	14.50	0

1 – presence of the specific product (susceptible), 0 – absence of the specific product (resistant)

our country and cause even higher yield losses and reduce grain quality, do not produce toxic substances. As reported by Steffenson (2003), in the field under variable weather conditions during infection and consecutive incubation period, mycelium infection can contribute to substantial changes in FHB development on cultivars with the same resistance level but different maturity. This effect can be observed in all field tests regardless of a method of inoculation. If a method for detached stem is used, most changes in these factors can be eliminated. Takeda and Heta (1989) indicated that if barley lines are inoculated either by using various methods or under various conditions in

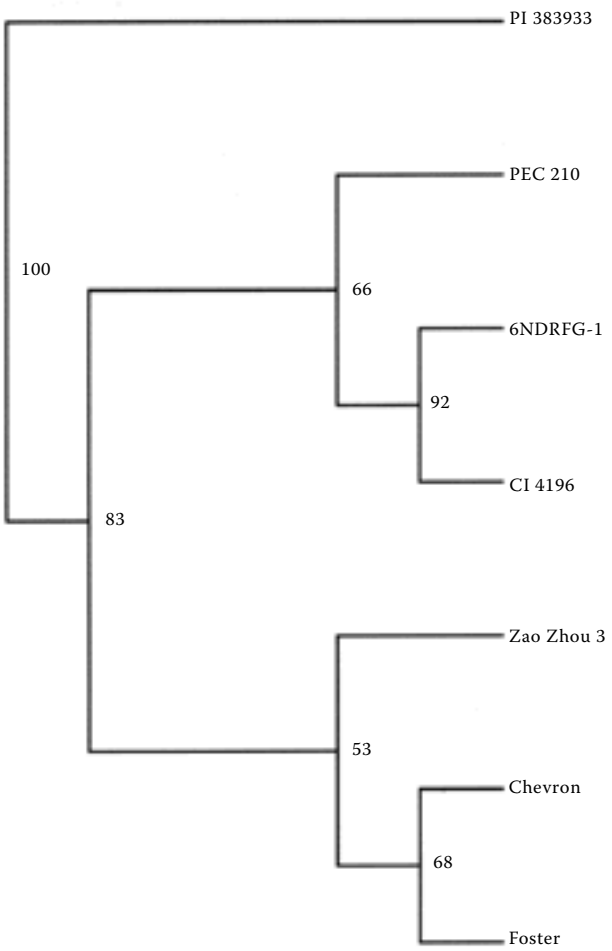

Figure 2. Dendrogram generated by UPGMA analysis calculated from RAPD markers using Nei and Li metrics showing relative genetic distance among analysed genotypes of spring barley

separate experiments, the final infections by fusaria do not highly correlate.

A set of parental genotypes of spring barley was tested with 80 RAPD markers. Out of this number, 72 tested RAPD markers were polymorphic in the examined genotypes (a total of 135 polymorphic alleles). The remaining 8 RAPD markers were monomorphic at least in two repeated analyses. RAPD marker H30 (sequence 5'GGA GTA ACG G 3') was found to enable to distinguish between very susceptible, resistant and/or moderately resistant parental genotypes based on the fragment of about 1300-bp size (Figure 1).

The specific marker H30 was screened in 23 DH lines derived from the cross of parental genotypes of spring barley (Table 2). This specific marker was found in 10 DH lines of spring barley (DH 1/1, DH 4/3, DH 5/1, DH 5/3, DH 5/12, DH 5/14, DH 5/16, DH 5/17, DH 7/1 and DH 9/2). The lowest disease severity in the field (13%) and the least DON content (3.30 ppm) were found for the line DH 4/2 derived from the cross of CI 4196 × Foster. The line DH 3/10 developed by the cross of PEC 210 × Foster showed a low percentage of grain infection in the laboratory assay (37%) as well as a low DON content (3.90 ppm). Such a level of FHB response also corresponded with the results of RAPD analyses (Table 2). DH lines were subjected to an analysis using selected RAPD primers (22–47 primers) enabling to differentiate individual parental components in crosses. Based on the presence of polymorphic products, a binary matrix was generated and phylogenetic trees were drawn using FreeTree and FreeView software and similarity coefficient according to Nei and Li (1979).

The tested genetic diversity of the chosen spring barley genotypes was expressed by a phylogenetic tree that enabled to differentiate two big clusters (Figure 2). The first one, entirely separate, consists of very susceptible genotype PI 383933. The other is split into two sub-clusters. The first one includes the genotype 6NDRFG-1 declared as moderately resistant and the resistant genotypes CI 4196 and PEC 210. The second sub-cluster consists of the moderately resistant genotype Zao Zhou 3 and the resistant cultivar Chevron. The same sub-cluster also includes Foster reported as a very susceptible cultivar to FHB.

The identical spring barley genotypes were analysed using the AFLP method. After screening 84 polymorphic markers, the genotypes were divided into the three clusters (Figure 3). The first cluster includes resistant cultivar Chevron and susceptible genotype PI 383933; the second cluster contains very susceptible cultivar Foster

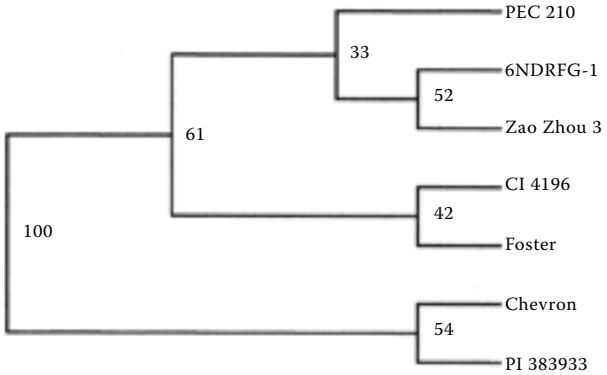

Figure 3. Dendrogram generated by UPGMA analysis calculated from AFLP markers using Nei and Li metrics showing relative genetic distance among analysed genotypes of spring barley

and resistant genotype CI 4196. The third cluster, which is genetically close to the second one, consists of resistant genotype Zao Zhou 3 and moderately resistant genotypes 6NDRFG-1 and PEC 210. The results obtained by AFLP and RAPD techniques are consistent only partly. Among others, it is due to different sensitivity, reproducibility and reliability of the methods employed. The AFLP method is known to be highly sensitive and more reliable as compared to RAPD method (Ovesná et al. 2002).

The cluster analysis reflects the pedigree of DH lines. If *in vitro* androgenesis is used in the breeding process, a significant factor is responsibility of individual genotypes. The differences in regeneration ability and regenerated green plants, the so-called genotypic specificity, are elucidated by different responsibility of parental genotypes (Machii et al. 1998). It means that genetic basis of the DH lines may not represent the whole scale of possible gene combinations. Hou et al. (1994) found that the DH lines of barley derived from F_1, F_2 and F_3 generations exhibiting higher responsibility had a higher proportion of genes from a responsible parent.

The marker H30 that allowed us to differentiate very susceptible parental genotypes of spring barley from the resistant ones is supposed to be converted into the SCAR marker (Sequence Characterised Amplified Regions) and to be used in selection of breeding materials. The conversion of these markers into SCARs by the development of more specific primers significantly improves the reproducibility and reliability of PCR assays and their utility for marker-assisted selection. The use of DNA SCAR markers is a very effective way of obtaining essential information about the genomic region around a given gene, selecting resistant lines in breeding programmes and ultimately isolating the gene of interest. The use of these markers in selection of resistant lines is also confirmed by Agrama et al. (2004).

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