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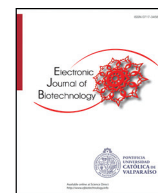
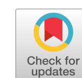


Research article

Production of wheat-doubled haploids resistant to eyespot supported by marker-assisted selection



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A R T I C L E I N F O

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A B S T R A C T

Background: Wheat is one of the most important crops cultivated all over the world. New high-yielding cultivars that are more resistant to fungal diseases have been permanently developed. The present study aimed at the possibility of accelerating the process of breeding new cultivars, resistant to eyespot, by using doubled haploids (DH) system supported by marker-assisted selection.
Results: Two highly resistant breeding lines (KBP 0916 and KBH 4942/05) carrying *Pch1* gene were crossed with the elite wheat genotypes. Hybrid plants of early generations were analyzed using endopeptidase *EpD1* and two SSR markers linked to the *Pch1* locus. Selected homozygous and heterozygous genotypes for the *Pch1*-linked *EpD1b* allele were used to produce haploid plants. Molecular analyses were performed on haploids to identify plants possessing *Pch1* gene. Chromosome doubling was performed only on haploid plants with *Pch1* gene. Finally, 65 DH lines carrying eyespot resistance gene *Pch1* and 30 lines without this gene were chosen for the eyespot resistance phenotyping in a field experiment.
Conclusions: Results of the experiment confirmed higher resistance to eyespot of the genotypes with *Pch1* in comparison to those without this gene. This indicates the efficiency of selection at the haploid level.
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1. Introduction

Wheat (*Triticum aestivum* L.) is the second most important crop in the world with the production of 750 million tons in 2016, 33% of which was grown in Europe [1]. Considering current yield trends, predicted population growth, and pressure of the environment, plant breeders must permanently develop new more effective varieties, i.e., of higher yield and resistance to environmental stresses. It is especially caused by the emergence of new races of pathogens and biotypes of pests as well as effects of climate changes [2,3]. The conventional wheat breeding is based on the selection of plants with desirable traits in the successive generations [4]. In the most commonly used pedigree breeding method, selection of plants starts in early generations. However, for traits of low heritability, selection is often postponed until the lines become more homozygous in later generations (F_5 or F_6). When the breeding lines become homozygous (or near-homozygous), they can be harvested in bulk and evaluated in replicated field trials. The entire process involves considerable time (5–10 years for elite lines to be identified) and expense [5]. Development of a new variety could take 8 to 12 years, including registration tests. To shorten this period, the conventional methods are often modified, e.g., by the single seed descent (SSD) technique supplemented with an embryo in vitro culture or by doubled haploid (DH) system [6,7,8,9,10]. Using DH technology, completely homozygous plants can be established in one generation, thus saving several generations of selfing, compared to conventional methods. DHs

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represent recombinant products of parental genomes in a completely homozygous state [11]. They can be propagated as true breeding lines, facilitating large-scale testing of agronomic performance over the years. Because of complete homozygosity, the efficiency of selection for both qualitative and quantitative characters increases, as recessive alleles are fixed in one generation and directly expressed. In wheat breeding, DH system has been widely applied. Numerous wheat cultivars of DH origin were released in Europe, USA, Canada, Brazil, and China [11,12,13].

Shortening of the breeding cycle, necessary to create homozygous plants with genes determining resistance to pathogenic fungi, is very important because pathogens quickly overcome the resistance of the plants; hence, there is a constant need of new varieties and lines with increased resistance.

Each wheat breeding method can be supported by the marker-assisted selection. Marker-assisted selection (MAS) involves selecting individuals based on their marker pattern (genotype), which contrasts with conventional breeding, where observable traits (phenotype) are evaluated. MAS has already being used in many breeding programs for backcrossing of major genes into elite parents [14,15,16].

Eyespot (strawbreaker food rot) is one of the most dangerous diseases of wheat (*T. aestivum* L.). It is caused by necrotrophic fungi *Oculimacula yallundae* (syn. *Tapesia yallundae*, Wallwork & Spooner) Crous and W. Gams and *Oculimacula acuformis* (syn. *Tapesia acuformis*; Boerema, R. Pieters & Hamers) [17,18]. Eyespot symptoms can reduce up to 50% of yield [19]. The main consequences of the infection are the premature ripening of grain, reduction of head length, reduction of thousand kernel weight, and finally lodging of wheat plants [20]. The most effective eyespot resistance gene is *Pch1* localized on the long arm of the wheat chromosome 7D [21]. It was identified in *Aegilops ventricosa* ($2n = 4x = 28$) [22,23] and transferred into wheat genome. There are several markers linked to the *Pch1* locus. One of them is an endopeptidase *EpD1b* allele. There are several SSR markers, which were used to identify the presence of the *Pch1* gene in the prebreeding germplasm, breeding lines and registered wheat cultivars [24,25,26,27, 28]. Some of them, *Xorw1*, *Xorw5*, and *Xorw6*, are accurate in predicting the presence or absence of the *Pch1* gene [29] but difficult to distinguish the allelic variation using basic horizontal electrophoresis methods on agarose gel. Moreover, a sequence-tagged-site marker *Xorw1*, derived from an oligopeptidase B encoding wheat expressed-sequence-tag showed complete linkage with *Ep-D1* [29]. Other *Pch1*-linked markers, i.e., *Xgwm37*, *Xbarc76*, *XustSSR2001-7DL*, *Xwmc14*, *Xbarc97*, and *Xcfd175* are flanking the *Pch1* locus. The closest one is *Xust2001-7DL* (3.9 cm) with easily identified allelic variations [29].

The main assumption of this study was to accelerate the breeding process of new wheat cultivars, resistant to eyespot, by using DHs system supported by MAS of wheat recombinants at the haploid and DH stage.

Our goal was to introduce the *Pch1* eyespot resistance gene into valuable cultivar Jantarka and two breeding lines of wheat. For that purpose, we have selected two highly resistant breeding lines with verified and effective *Pch1* gene [20,27], which were crossed with the elite genotypes. Marker analyses were used for the selection of haploids carrying the *Pch1* gene. DH lines with *Pch1* were chosen for the eyespot resistance phenotyping in a field experiment.

2. Materials and methods

2.1. Plant material

Material for the studies covered six cross combinations of winter wheat: K_1 — KBP0916 × Jantarka, K_2 — KBP0916 × STH9014, K_3 — SMH8892 × KBH4942, K_4 — KBP0916 × POB32408, K_5 — D 323/07 × Patras, and K_6 — D 414/07-4 × KWS Ozon. In K_1–K_4 combinations, two parental lines KBH4942/05 and KBP0916 (from Plant Breeding Małopolska Ltd.) were identified to possess *Pch1* gene, whereas the SMH 8892 (from Plant Breeding Smolice Ltd.) and POB 32408 (from Plant Breeding Poznań Ltd.) and Polish cultivar Jantarka were characterized by good technological properties. Cross combinations K_5 and K_6 were produced by the crossing of high-quality breeding lines D323/07 and D414/07-4 (from Plant Breeding Danko Ltd.) with cultivars Patras and Ozon. F_2 hybrids of K_1–K_4 cross combinations and cultivar Rendezvous as the resistance control were analyzed using SSR markers and endopeptidase assay for the identification of plants carrying the *Pch1* gene.

2.2. Haploid production

Haploids were produced by wheat × maize crossing using the standard procedure. Briefly, spikes were manually emasculated and pollinated with fresh pollen of maize (*Zea mays* L.; cv. Waza). Pollinated spikes were treated with 2,4-dichlorophenoxyacetic acid (2,4-D). Immature embryos were dissected from seeds 15–18 d after pollination and cultured in vitro on B5 medium [30] in tubes. Haploid plants were vernalized for 8 weeks. Subsequently, haploids were treated with colchicine solution for chromosome doubling [10].

2.3. Identification of the Pch1 gene

2.3.1. SSR analysis

The parental genotypes, hybrids, haploids, DHs, and cultivar Rendezvous (resistance control) were screened using publicly available SSR markers (Table 1) that were polymorphic between parental genotypes. Markers were chosen to provide a coverage of the terminal region of the long arm of chromosome 7D. Primer set for *Xbarc97* was used from Beltsville Agricultural Research Station [31,32] and *XustSSR2001-7DL*, which is linked to *Pch1* locus transferred from *A. ventricosa* [25]. Total genomic DNA was extracted from 14 d old seedlings using Plant DNA Purification Kit (Eurx, Poland). The markers were amplified according to previous reports [25,27,29]. The PCR profile was modified with reference to standard protocol and consisted of denaturation at 94°C for 10 min, followed by 35 cycles of 94°C for 30 s, 55°C (*XustSSR2001-7DL*) or 58°C (*Xbarc97*) for 30 s and 72°C for 1 min, followed by final extension for 10 min at 72°C and a soak temperature of 4°C. The products of amplification were separated using 2–3% agarose (SIGMA) gel (1× TBE buffer, 5 h at 100 V) and visualized with ethidium bromide.

2.4. Endopeptidase assay

The endopeptidase assay was conducted using the same leaf tissue that has been used for the SSR analyses. The enzyme was extracted by grinding the two-week-old leaves using a plexiglass bar in 10 μl of 0.025 M glycyl-glycine buffer (pH 7.4; SIGMA). To load samples, paper strips were soaked in the enzyme extract for each genotype and inserted into the gel. The 10% starch (SIGMA) gel was run at 4°C at 200 V. After electrophoresis, the gel was incubated in the dark at 37°C for 30 min with 0.5% solution of low-melting agarose containing 2.56 mg Fast Black K Salt (SIGMA) and 1.12 mg N-α-Benzoyl-DL-Arginine-LB-Naphthylamide (SIGMA) in 0.1 M Trizma maleate (SIGMA) – NaOH [28].

Table 1
Sequences for molecular markers associated with the *Pch1* gene used in the study.

Marker	Primer sequence 5′ → 3′
XustSSR2001-7DL F	CAT CGT GTG GCC AAC TTG TT
XustSSR2001-7DL R	TTC CTC GTG TCT AGT GTC TC
Xbarc97 F	GCG CCA ACT ACG GAG CTC GGA GAA T
Xbarc97 R	GCA GGA TCA AAC GTA GCC ATG GTG

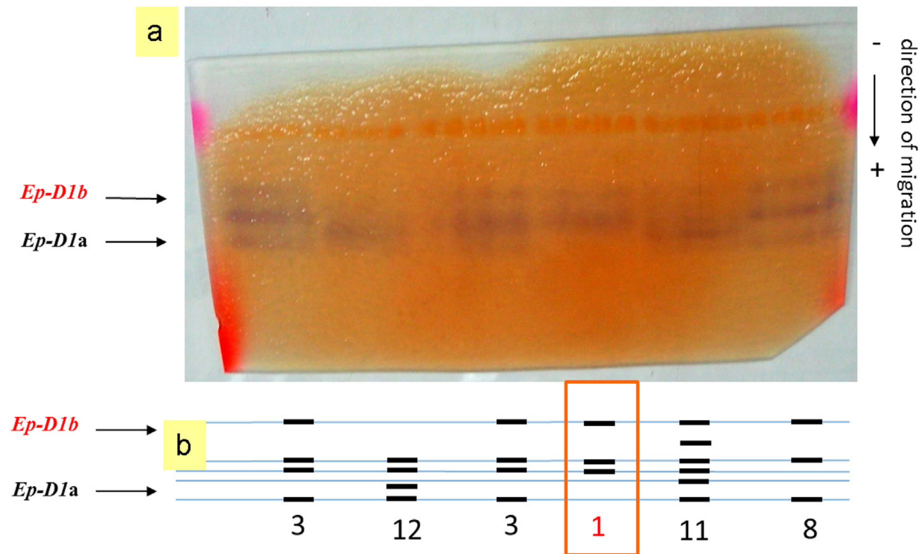

Fig. 1. Isozyme patterns of endopeptidase *EpD* system in six wheat genotypes, five plants each. a) photograph of the starch gel after electrophoresis, b) schematic graph. *EpD1a*, *EpD1b* – endopeptidase alleles. Banding pattern with 3 stripes (type "1") identify *Pch1* gene. Other types of banding patterns indicate lack of *Pch1* gene.

2.4.1. Phenotyping for the eyespot resistance

Sixty-five DH lines developed from K_1–K_4 combinations (carrying the *Pch1* gene), 30 lines developed from K_5–K_6 combinations, and all DHs possessing a sufficient number of kernels were selected for field experiment established in the second year of studies for the verification of the resistance to eyespot. Besides DH lines, parental genotypes and resistant cultivar rendezvous were incorporated into the experiment.

A field experiment was performed in 2016 at Cerekwica near Poznań (52°13′16″N 16°41′30″E; Poland). The experiment was conducted in 3

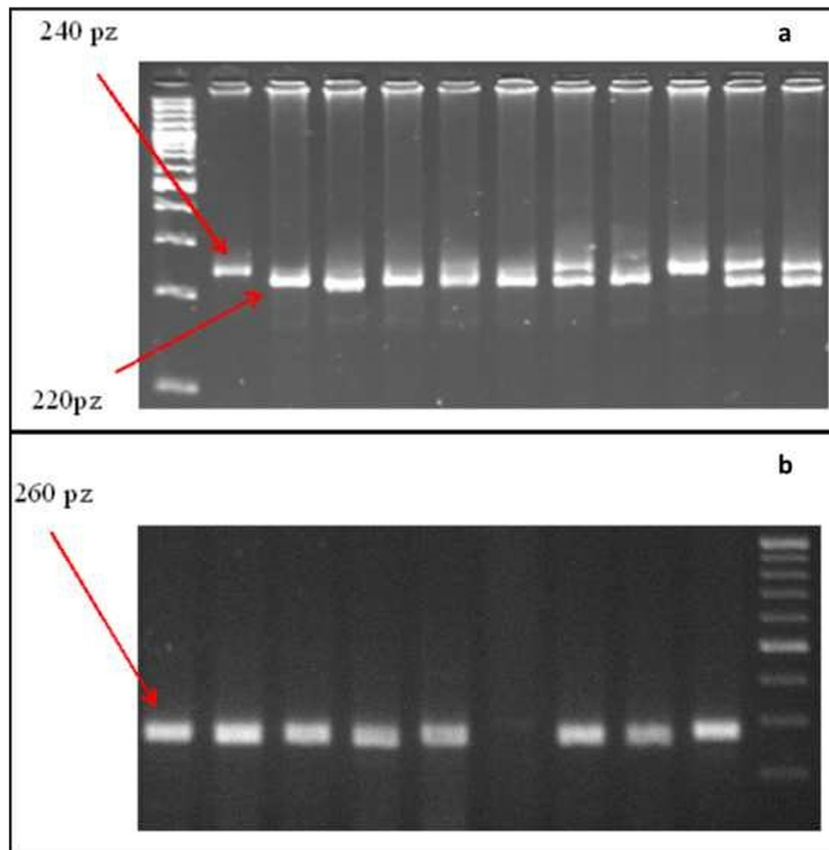

Fig. 2. (a) PCR reaction products after electrophoresis in UV light for marker *XustSSR2001-7DL*. Products of 240 bp in size characterize plants with the *Pch1* gene. Products of 220 bp in size characterize plants without the *Pch1* gene. Both products indicate heterozygous plants; (b) PCR reaction products after electrophoresis in UV light for marker *Xbarc97*. Lack of band characterizes plants with the *Pch1* gene. Products of 260 bp in size characterize plants without the *Pch1* gene.

Table 2
Results of *Pch1 locus* identification using *EpD1*, *XustSSR2001-7DL*, and *Xbarc97* markers in hybrid plants and selected haploids derived from hybrids.

Cross combination/plant number	*Pch1* status of hybrid plants: H – homozygous h – heterozygous	Haploid plant no.	*EpD1*allele	Amplification products (bp)	
				XustSSR2001-7DL	*Xbarc 97*
K_1/1	H	1	b	240	0
		2	b	240	0
		3	b	240	0
		4	b	240	0
K_1/2	H	5	b	240	0
		6	b	240	0
K_1/3	h	7	a	220	260
		8	a	220	260
		9	a	220	260
		10	a	220	260
		11	b	240	0
		12	b	240	0
K_1/4	H	13	b	240	0
		14	b	240	0
		15	b	240	0
		16	b	240	0
		17	b	240	0
K_2/1	H	18	b	240	0
		19	b	240	0
		20	b	240	0
K_2/2	H	21	b	240	0
		22	b	240	0
		23	b	240	0
		24	b	240	0
		25	b	240	0
K_2/3	H	26	b	240	0
		27	b	240	0
K_2/4	H	28	b	240	0
		29	b	240	0
K_2/5	H	30	b	240	0
		31	b	240	0
		32	b	240	0
K_2/6	H	33	b	240	0
K_2/7	h	34	a	220	260
		35	b	240	0
K_2/8	H	36	b	240	0
K_2/9	h	37	a	220	260
K_3/1	h	38	a	220	260
K_3/2	H	39	b	240	0
K_3/3	H	40	b	240	0
K_3/4	H	41	b	240	0
		42	b	240	0
		43	b	240	0
K_3/5	h	44	a	220	260
K_3/6	h	45	b	240	0
		46	a	220	260
		47	b	240	0
		48	b	240	0
K_4/1	h	49	b	240	0
		50	b	240	0
		51	b	240	0
		52	a	220	0
K_4/2	H	53	b	240	0
K_4/3	h	54	a	220	260
K_4/4	H	55	b	240	0
		56	b	240	0
		57	b	240	0
K_4/5	H	58	b	240	0
		59	b	240	0
		60	b	240	0
		61	b	240	0
		62	b	240	0
		63	b	240	0
		64	b	240	0
K_4/6	H	65	b	240	0
		66	b	240	0
Rendezvous (control)	H	RV	b	240	0

replications. Each replication of each line was composed of a one-meter-long row with 30 seeds. The same three replications were used to control experiment (without inoculation).

Plant material was inoculated by spraying plants at BBCH 31–32 scale with a fresh-made conidial-mycelium suspension of *Oculimacula acuformis* and *O. yallundae* (1:1 ratio, 4 × 106 spores/ml) in April.

Table 3
Results of K-index and coefficient of variation parameters, determining resistance of DH lines with and without the *Pch1* gene, evaluated in the field inoculation test.

No.	Cross combinations	Inoculation test				Control
		Mean from 3 replication K = index score	Standard error	Coefficient of variation (%)	Range	Mean from 3 replication K = index score
DH lines with the Pch1 gene						
K_1	KBP 0916$^{(}$(*Pch1*) × cv. Jantarka	0.21	0.018	55.95	0.17–0.32	0.25
K_2	KBP 0916(*Pch1*) × STH 9014	0.22	0.017	60.27	0.12–0.33	0.26
K_3	SMH 8892 × KBH 4942^{+}(*Pch1*)	0.20	0.015	36.14	0.12–0.27	0.24
K_4	KBP 0916^{+} *Pch1* × POB 32408	0.23	0.013	42.34	0.08–0.37	0.29
DH lines without the Pch1 gene						
K_5	D 323/07 × Patras	0.89	0.026	17.88	1.03–1.80	0.33
K_6	D 414/07-4 × KWS Ozon	0.94	0.024	18.44	0.78–1.17	0.28

Eyespot symptoms were observed on mature plants at BBCH 71–92. The evaluation was performed on 20 plants from each replicate of each line and control (120 leaf sheaths for each line). The percent of infected leaf sheaths was determined, and the leaf sheath infection index was calculated. The level of the leaf sheath infection was evaluated using I–IV scale, and the results were presented as a mean from each replication according to the K-index formula [26]: I — no symptoms, II — less than 50% of leaf sheaths surface infected, III — over 50% of leaf sheaths surface infected, IV — 100% of leaf sheaths surface infected, rotten tissue noted:

$$K = \frac{[n(II)x0,25] + [n(III)x0,75] + n(IV)}{n(I + II + III)},$$

where n is the number of evaluated stalks.

2.4.2. Statistical analysis

The K-index values from each replication of K_1–K_6 DH lines were averaged. Analysis of variance (ANOVA) was used to examine the differences between distinguished groups of DH lines with regard to the K-index value. All hypotheses about the equality of genotype groups were tested at P = 0.05 and 0.01 significance level. With the rejection of the hypothesis of no differences between groups, the least significant difference test ($LSD_{0.05}$ and $LSD_{0.01}$) was used for the planned pair comparisons and F-test for studying of the significance of various contrasts between groups [33]. Additionally, examined DH lines were divided into homogeneous groups, in such a way that variability (sum of squares of deviations) within a group was as small as possible, while the variability among groups was maximized.

Table 4
Estimation of differences in K-index parameter between DH lines derived from particular cross combinations, determined in the field inoculation test.

No.	Contrast	Estimation of contrast	F stat.
1	K_2–K_1	0.007	0.07
2	K_3–K_1	−0.016	0.25
3	K_4–K_1	0.009	0.13
4	K_3–K_2	0.010	0.09
5	K_4–K_2	0.003	0.01
6	K_4–K_3	0.067	0.04
7	K_6–K_5	0.040	2.04
8	K_1–K_5	−0.670	539.62^{xx}
9	K_2–K_5	−0.670	599.83^{xx}
10	K_3–K_5	0.680	408.49^{xx}
11	K_4–K_5	0.680	568.51^{xx}
12	K_1–K_6	−0.707	701.62^{xx}
13	K_2–K_6	−0.714	789.95^{xx}
14	K_3–K_6	−0.723	507.95^{xx}
15	K_4–K_6	−0.717	740.82^{xx}

K_1 (with *Pch1* gene): KBP 0916 × cv. Jantarka.
K_2 (with *Pch1* gene): KBP 0916 × STH 9014.
K_3 (with *Pch1* gene): SMH 8892 × KBH 4942.
K_4 (with *Pch1* gene): KBP 0916 × POB 32408.
K_5 (without *Pch1* gene): D 323/07 × Patras.
K_6 (without *Pch1* gene): D 414/07-4 × KWS Ozon.
^{xx} $P < 0.01$.

3. Results and discussion

In the experiment, 604 haploid plants were derived from K_1–K_6 cross combinations. Chromosome doubling resulted in the production of 458 DH lines. Haploid plants were screened using a set of markers linked to the *Pch1* locus (*EpD1, XustSSR2001-7DL,* and *Xbarc97*). On this basis, 65 DH lines with the *Pch1* gene were chosen for the eyespot resistance phenotyping in a field experiment along with 30 DH lines obtained from K_5 and K_6 combinations (without *Pch1* gene) treated as a negative controls.

It was reported that *Pch1* locus co-segregated with the *Ep-D1b* allele [34] with no recombination between *Ep-D1* and *Pch1* [21]. Thirteen isozyme patterns were detected in analyzed genotypes. Presented results confirmed the information about the presence of the *Pch1* gene in wheat genotypes KBP 0916 [27] and KBH 4942/05 [20], reported previously. The band associated with the *Ep-D1b* locus and the middle band, without a band for *Ep-D1a* (isozyme pattern 1), is related with the complete resistance to eyespot disease (Fig. 1).

Moreover, the SSR marker analysis confirmed the results obtained in the endopeptidase assay. According to Groenewald et al. [25], the *XustSSR2001–7DL* marker is closely linked to the endopeptidase locus *Ep-D1*. This marker resulted in 220 bp band associated with *Ep-D1a* and a 240 bp band associated with *Ep-D1b* (Fig. 2a). Those results were in line with the *Xbarc97* marker analyses. This marker is localized in the 7DL chromosome and determine the region of *Pch1* locus introgression. In genotypes without a *Pch1* locus, this marker resulted in a 260 bp product (Fig. 2b), while no product was amplified for *Pch1* genotypes, as follows: Rendezvous, KBP 0916, and KBH 4942/05.

Unfortunately, this SSR marker is dominant, failing to amplify the *A. ventricosa* allele, and suitable only for screening homozygous material in breeding programs. On the other hand, it shows the region of the 7DL chromosome, where the chromatin of *A. ventricosa* is incorporated. Thus, the most informative way to predict the presence and the chromosome localization of the *Pch1* locus is to analyze all three markers (*EpD1*, *XustSSR2001-7DL* and *Xbarc97*) for homogeneous plant material, but for heterogeneous germplasm only *EpD1* and *XustSSR2001-7DL* are informative.

The first step of this study was to select plants with the *Pch1* locus from F_2 generation using endopeptidase assay, *XustSSR2001-7DL*, and *Xbarc97* marker analyses. Within the breeding process, DH lines have been usually derived from F_1 hybrids, although some breeders prefer to induce DH lines from later generations. Haploids induction in the F_2 generation is an option because lines originated from F_3 generation gametes had passed through another recombination cycle. But, on the other hand, the F_3 offspring seed number is larger than in F_1 generation. However, Choo et al. [35], comparing DH and SSD methods, showed that there was no difference in the sample of recombinants. In our study, we chose 25 F_2 plants from four combinations (K_1, K_2, K_3, and

Table 5
Designation of the two homogeneous groups of DH lines on the basis of presence or lack of the *Pch1* gene and K-index parameter.

Group	Group mean of K-index	DH lines number	Combinations
1	0.92	13, 20, 4, 17, 18, 3, 6, 1, 11, 30, 16, 7, 21, 25, 28, 14, 19, 12, 5, 10, 24, 8, 9, 27, 2, 15, 23, 29, 22, 26	Nos 1–13: K_5 (D 323/07 × Patras) Nos 14–30: K_6 (D 414/07-4 × KWS Ozon
2	0.22	66, 80, 91, 50, 51, 53, 92, 68, 73, 57, 56, 85, 58, 32, 89, 49, 65, 43, 35, 78, 41, 40, 47, 70, 48, 45, 38, 75, 72, 59, 94, 88, 39, 71, 37, 44, 74, 42, 34, 33, 93, 76, 84, 90, 69, 55, 82, 83, 46, 62, 79, 86, 77, 52, 87, 64, 67, 81, 95, 61, 35, 60,54, 63, 31	Nos 31–40: K_1 (KBP 0916 $^+$*Pch1* × cv. Jantarka) Nos 48–68: K_2 (KBP 0916$^+$*Pch1* × STH 9014) Nos 69–77: K_3 (SM 8892 × KBH 4942 $^+$*Pch1*) Nos 78–95: K_4 (KBP 0916 $^+$*Pch1* × POB 32408)

K_4), which were produced by crossing two breeding lines with the *Pch1* gene. From the 25 plants, we selected 17 plants, which contained a single band for *EpD1b* allele and a single, 240 bp product for *XustSSR2001-7DL* marker (Table 2). Moreover, we selected 8 genotypes, which carried the band associated with *Ep-D1b* locus and the middle band and furthermore, two products of 240 bp and 220 bp for *XustSSR2001-7DL* marker (Table 2). The same variants of heterozygous plants were observed in previous reports [20,28]. Those 25 plants were used for haploid production. As it was expected, two groups of haploid plants were obtained, with and without the *Pch1* gene. All haploids obtained from *Pch1* homozygous plants carried alleles associated with the *Pch1* locus: *EpD1b*; 240 bp product for *XustSSR2001-7DL* and lack of product of *Xbarc97* marker amplification. Table 2 shows the results of the marker analyses of these haploids, from which DH lines were obtained and subsequently tested in the field experiment. According to the results of molecular analyses, 65 DH lines with the *Pch1* locus were chosen for the eyespot resistance phenotyping in a field experiment. The experiment was performed on DH lines, for which a sufficient number of kernels were obtained to establish a field inoculation and control treatments. The remaining lines were allocated for multiplication.

The leaf sheath infection characterized by the mean of K-index was low (0.20–0.23) for the DH lines originated from the K_1–K_4 combinations and carrying the *Pch1* gene. Considering single samples, the K-index range was 0.08–0.37 for *Pch1* bearing plants (Table 3). K-index for parental genotypes with *Pch1* gene (KBP 4942 and KBP 0916) ranged from 0.05 to 0.10 but for parental genotypes without *Pch1* gene (SMH 8592 and Jantarka) ranged from 0.61 to 0.64 (Table 3). In the group of DHs from the combinations K_5 and K_6 (without *Pch1* gene), K-index was higher and ranged from 0.89 to 0.94. Further, the K-index for the resistance cultivar Rendezvous, used as a control line, was low (0.06).

In summary, we have obtained 65 DH lines of winter wheat carrying eyespot resistance gene *Pch1*, which were verified in the field trials in the second year of the study. Moreover, this study shows that MAS applied for the DH lines production can be useful in terms of the breeding process acceleration. It is also worth to underline that a set of markers used in this study are crucial for the selection of homozygous plant material with the known region of *Pch1* locus introgression.

In our study, we have found that there is a possibility to create the homozygous material with the desired gene (*Pch1*), throughout the material selection with the targeted gene, at the haploid level and creating the DH lines from haploids carrying the *Pch1* gene only. This study shows that the MAS, performed at the haploid stage before DH lines production, can significantly and effectively shorten the breeding process. This reduces the costs of creating new varieties as well as shortens the cycle of their generation, which is very beneficial from the point of breeding.

The ANOVA showed significant differentiation ($P < 0.01$) of the studied DH lines in K-index values. Hence, we compared the groups of DHs originating from K_1–K_6 cross combinations. Estimation of the differences between groups and F statistic, for contrasts, indicates that these differences were not significant between DHs from K_1, K_2, K_3, and K_4 and between K_5 and K_6 but highly significant between groups of lines derived from combinations with and without *Pch1* gene (Table 4), which is also reflected by dividing DHs into homogeneous groups (Table 5). The K-index mean for *Pch1*-carrying DH lines was 0.22, which was lower than 0.92 calculated for DH lines without *Pch1* locus.

Conflict of Interest Statement

The authors have no conflicts of interests to declare.

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