

Molecular Identification of *Pm4* Powdery Mildew Resistant Gene in Oat

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Abstract

The selection of specific plants with desirable traits supported by molecular markers is one of the most important tools in modern breeding programs, which lead to reduce time and cost of selection. The aim of presented study was identification of dominant markers associated with *Pm4* powdery mildew resistant gene in oat. To identify dominant silicoDArT markers for *Pm4* gene, F₂ mapping population 'Av1860' × 'Fuchs' were analyzed using DArTseq methodology. Among obtained 46 230 silicoDArT markers, 126 markers were high correlated with resistance to powdery mildew in oat conditioned by *Pm4* gene. Among selected markers, 48 sequences have been chosen for potential conversion into specific STS markers. Finally, only 20 were suitable for primer design. As a result, 5 converted markers amplified expected products in resistant bulks, 3 of them segregated according to resistance in the whole population and showed high correlation coefficient between marker and phenotype observation. Converted markers based on PCR could be used for identification of *Pm4* gene in oat. Obtained results confirm the possibility of converting silicoDArT markers into PCR-based technique, which can be used in marker assisted selection (MAS).

Keywords: *Avena sativa*; *Blumeria graminis*; molecular markers; resistance

Introduction

Powdery mildew caused by *Blumeria graminis* DC. f.sp. *avenae* Em. Marchal. is one of the most important foliar disease of cultivated oat is. This worldwide disease appears every year and causes big crop losses (Lawes and Hayes, 1965; Jones, 1977; Schwarzbach and Smith, 1988; Sebesta *et al.*, 1991; Clifford, 1995; Aung *et al.*, 1977; Roderick *et al.*, 2000). Large genetic changeability and ability to generate new forms by mutations and DNA recombinations makes this pathogen easily adaptable to new conditions (Bennet, 1984; Bayles, 1997). The use of fungicides is the easiest method of protection against pathogen attack, but cultivated oat is a low-input crop which grown on less productive lands, so in this case, breeding resistant cultivars seems to be more economical and more environmental friendly method for controlling and limitation occurrence of oat powdery mildew. Till date, 8 resistant genes against powdery mildew have been described in oat. Hsam *et al.* (1997, 1998), Okoń (2012), Okoń *et al.* (2016) demonstrate that only 3 of them (*Pm1*, *Pm3* and *Pm6*) were used in worldwide breeding programmes. However resistant to powdery mildew controlled by these genes is breaking down by the existing races of powdery mildew (Okoń, 2015). Nowadays the most effective sources against powdery mildew in oat is *Pm4* resistant gene (Okoń, 2015).

Pm4 was derived from the diploid oat species *A. barbata* accession Cc4897 (Rajhathy, 1963; Aung and Thomas, 1978). Resistance controlled by this gene was identified in a field nursery in 1978 (Jones and Jones, 1979), also *Pm4* gene has remained effective at all EODN (European Oat Disease Nursery) nurseries outside Britain (Sebesta *et al.*, 1997). Okoń (2015) confirm that resistance conditioned by *Pm4* gene is still effective in central Europe. High level of resistance continuing in recent years suggested that *Pm4* should be used in breeding programmes to increase level of resistance to powdery mildew in cultivated oat.

The fundamental basis of plant breeding is the selection of specific plants with desirable traits. Marker-assisted selection (MAS) is one of the most important tools in modern breeding programs, which lead to reduce time and cost of selection. This method based on identification of molecular markers associated with particular traits, and combines the knowledge about a genotype and phenotype of the analyzed plants. MAS increased the efficiency of conventional selection methods and offer the possibility to identify genotypes with desired traits in seedling stage and irrespective of environmental conditions (Collard *et al.*, 2005; 2008). This advantage of MAS is very important in selection of genotypes resistant to fungal diseases. Using molecular markers instead of physiological tests lowers the cost and time of selection (Wight *et al.*, 2005; Yu and Herrmann, 2006; Chhetri *et al.*, 2017).

The presented work is the first report about the effort of the development of an effective method for the identification of the powdery mildew resistance gene *Pm4* in oat. The aim of presented study was identification of dominant DArTseq markers associated with *Pm4* resistant gene and conversion of these markers into simple PCR-based method.

Materials and Methods

Plant material

The subject of the study was segregating population derived from cross between line 'Av1860' possessing *Pm4* powdery mildew resistant gene with susceptible oat cultivar 'Fuchs'. 157 individuals of F₂ population were phenotyped based on host-pathogen test. After tests all individuals were planted on experimental plot. F₃ generation seeds were collected from every F₂ individuals and tested using host-pathogen methodology. Obtained observations allows select both homozygous-resistant and homozygous-susceptible individuals for molecular analysis.

Resistance tests

Resistance of the individuals in F₂ and F₃ populations was estimated based on 2 single spore powdery mildew isolates avirulent to *Pm4* gene. Selected isolates were collected from different parts of Poland. Single spore isolates were obtained according to description in our previous publication (Okoń and Kowalczyk, 2012a). Host-pathogen tests were carried out on the first leaves of 10-day-old seedlings according to method described by Okoń and Kowalczyk (2012a). The effect on the leaves was determined ten days after inoculation. Reactions to the isolates were grouped into two classes: resistant, from 0 to 20% infection relative to 'Fuchs', and susceptible where the degree of infection exceeded 20% (Hsam *et al.*, 1997). The segregation ratio of the F₂ and F₃ populations were analyzed using Chi-Square test for goodness of fit.

DNA extraction

Genomic DNA from all F₂ individuals was extracted from fresh 10-day-old leaves using DNeasy Plant Mini Kit (Qiagen). DNA integrity and quality were evaluated by electrophoresis on 1.5% agarose gel. DNA concentration was determined with NanoDrop2000 spectrophotometry and normalized to 50 ng µl⁻¹.

High-throughput genotyping using the DArT-SeqTM method

A high-throughput genotyping method based on DArTseq technology was employed to genotype F₂ population. Restriction enzymes *PstI* and *HpaII* were selected for genomic complexity reduction. The silicoDArT markers were scored using DArTsoft as a binary data (0/1), several quality parameters such as call rate, polymorphism information content (PIC) and reproducibility were calculated.

Conversion of silicoDArT markers to PCR-based assay

A SilicoDArT markers highly correlated with phenotype observations were chosen for further analysis.

Markers sequences associated with resistance to powdery mildew, were analyzed in CLC Main Workbench software version 7.8 to identify primer pairs for their amplification. The main criteria for primer design were as follows: primer size 16-22 bp, 40-60% GC rich (optimum 50%), minimum annealing temperature 55 °C (optimum 60 °C), product size >40bp.

Specific PCR

Reaction mixtures in a final volume 10 µl, consisted 60 ng of total genomic DNA, 20-40 µM each of PCR primers (quantity was tested for each primers), 0.1 mM dNTPs, 1.5-2.5 mM MgCl₂ (quantity was tested for each primers), 1 × reaction buffer and 0.5 U Taq Polymerase ThermoFisher). PCR was conducted on T1Biometra thermocycler. The following profile: 95 °C–7 min, 35 × 95 °C–30 s, 56 °C–30 s, 72 °C–30 s with final elongation 72 °C–5 min was used. The PCR products were separated on 2.5% agarose gels containing EtBr in TBE buffer at 140 V for 1 h.

Marker validation

Converted silicoDArT-based markers were tested in all individuals of 'Av1860' × 'Fuchs' segregating population. Segregation evaluated based on silicoDArT and their converted counterparts were compared for congruency. Spearman rank correlation coefficients between converted markers and resistance of plant materials were calculated using Statistica software 13.1 (StatSoft, 2017). The significance of association between evaluated molecular profiles and resistant and susceptible plants was assessed via Pearson's Chi Square test (Bewick *et al.*, 2004) using Statistica software 13.1 (StatSoft, 2017).

Results

Analysis of resistance segregation

'Av1860' × 'Fuchs' F₂ population were tested in 2 independent host-pathogen tests based on 2 different powdery mildew isolates. Segregation of the F₂ plants analyzed population deviated significantly from the ratio 3:1. Number of resistant and susceptible individuals was very similar for all used powdery mildew isolates: 124, 119 resistant and 34, 39 susceptible plants were identified in test based on Kopaszewo 2015(1) pk and Czesławice 2015 (2) isolates respectively (Table 1).

The segregation for resistance to powdery mildew in F₃ mapping population approximated a 1:2:1 ratio corroborating single-gene segregation (Table 1). Obtained results allow choosing resistant and susceptible homozygous form analyzed population.

To identify silicoDArT markers for *Pm4* resistant gene to oat powdery mildew F₂ mapping population 'Av1860' × 'Fuchs' were analyzed using DArTseq methodology. A total of 46230 silicoDArT markers were identified in mapping population; 10 564 markers were polymorphic among the 157 individuals of F₂ population. The polymorphism information content (PIC) ranged from 0.013 to 0.500 with an average 0.374. 236 markers had a very low PIC (a value less than 0.05) which means that this markers characterized very low polymorphism. The call rate ranged from 79.2 to 100% with an average of 92.2% (Table 2).

Among obtained silicoDArT markers 126 were highly correlated with resistance to powdery mildew. Patterns of selected markers were very similar and allow distinguishing resistant and susceptible genotypes in mapping population. Segregation of all silicoDArT markers associated with *Pm4* gene, across the population match to the ratio 3:1 (data not shown). Among these markers 48 sequences have been selected for potential conversion into specific markers, but only 20 were suitable for primer design. Two pairs of primers were designed for each sequence.

In first step all designed markers were tested on parental genotypes and bulks of resistant and susceptible individuals. 5 primer pairs amplified expected products in resistant bulks and 'Av1860' line. The rest of primer pairs produced amplicons in all tested genotypes.

Five primer pairs were tested on resistant and susceptible homozygous individuals from 'Av1860' × 'Fuchs' population. Only 3 primer pairs amplified expected patterns: product present in resistant and absent in susceptible genotypes (Table 3, Foto 1). These primers were verified on parental lines and 144 individuals of mapping population 'Av1860' × 'Fuchs'. Marker PM4-2 was

observed in 112 among 146 analyzed genotypes. Expected amplification product was present in 103, among 108 resistant individuals and in 'Av1860' parental line and in 8 genotypes characterized as a susceptible in host-pathogen tests. This marker was highly correlated with resistance conditioned by *Pm4* gene, correlation coefficient assumed value $r=0.759$ ($p>0.0001$). Second marker named Pm4-6 was observed in 112 analyzed genotypes. 102 individuals characterized as a resistant and 10 characterized as a susceptible possess this marker. Spearman rank correlation coefficients between marker and phenotype observation was also very high ($r=0.705$, $p>0.0001$). Third successfully converted marker Pm4-9 was present in 99 individuals, 96 resistant and 3 susceptible genotypes possessed this marker. Correlation coefficient between this marker and phenotype observation assumed value $r=0.744$ ($p>0.0001$). The Chi square test indicated a significant association between individuals characterized as resistant and susceptible and converted molecular markers. The value of the χ^2 test ($p=0.000$) was 84.196 for Pm4-2 marker, 72.690 for Pm4-6 and 80.919 for Pm4-9.

Table 1. Seedlings response and segregation ratio of F₂ and F₃ families derived from 'Av1860' × 'Fuchs' cross inoculated with different *Blumeria graminis* DC. f.sp. *avenae* Em. Marchal isolates

Powdery mildew isolates	F ₂ population ('Av1860' × 'Fuchs')				F ₃ population ('Av1860' × 'Fuchs')				
	Resistant	Susceptible	Chi-square	P-value	Resistant	Segregating	Susceptible	Chi-square	P-value
Kopaszewo 2015(1) pk	123	35	0.544	0.30	57	72	35	4.902	0.05
Czesławice 2015 (2)	119	39	0.000	0.99	51	68	39	5.492	

Table 2. Quality parameters of the silicoDArT markers used to analyze 'Av1860' × 'Fuchs' mapping population

Parameter	Maximum value	Minimum value	Average value
Polymorphism information content	0.500	0.013	0.374
Reproducibility (%)	1.00	0.941	0.994
Call rate (%)	100	79.2	92.2
One ratio	0.814	0.006	0.734

Table 3. SilicoDArT markers successfully converted into SCAR

SilicoDArT marker	SCAR primers	Primers sequence	Primer TM	SCAR length	Segregation of converted markers	Correlation with phenotype observation
10575906	Pm4-2 F1	CAGCCGTGAGGGTGAAC	60	46	0.147 ($p=0.50$)	0.759
	Pm4-2R	GCTCTCCTTGTAAGGTCCA	60			
5420825	Pm4-6F	GCCAAACCTGATAGTGACCAA	60	47	0.147 ($p=0.50$)	0.705
	Pm4-6R	TACGCCAAGCGTGTCAGTTA	60			
22075478	Pm4-9F	GCAGGTGTGTCTGAATGTCG	60	68	4.461 ($p=0.02$)	0.744
	Pm4-9R	GGGCATCAGGCATATCG	58			

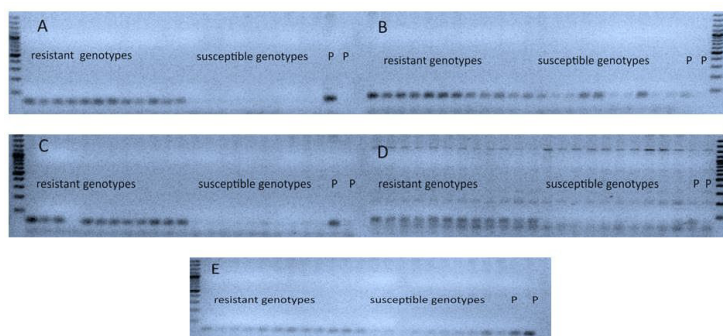


Fig. 1. Molecular profiles obtained with Pm4-2 (A), Pm4-3 (B), Pm4-6(C), Pm4-9 (D), Pm4-16 (E) primer pairs for resistant and susceptible individuals and parental line of 'Av1860' × 'Fuchs' population

Discussion

One of the most important purpose of many plant breeding programs is to prevent decreases in yield due to fungal diseases. One of the most harmful oat fungal disease in powdery mildew caused by *Blumeria graminis* DC. f.sp. *avenae* Em. Marchal. Annual crop losses from mildew infections are estimated to range from 5-10% every year up to 40% in the years with high disease pressure (Lawes and Hayes, 1965; Jones, 1977; Roderick and Clifford, 1995). This is known that the most economical and environment friendly method for controlling fungal diseases is breeding cultivars with genetically conditioned resistance. In oat, identification of genotypes possessing some resistance to powdery mildew is based on the host-pathogen tests. These tests based on observation the effect of fungus infection on the studied form with unknown resistance and comparison this reaction with the effect appearing on lines with known resistant genes (Hsam *et al.*, 1997; 1998; Kowalczyk *et al.*, 2004; Yu and Herrmann, 2006; Okoń and Kowalczyk, 2012a; Okoń *et al.*, 2016). It all makes it very time and work-consuming. Nowadays molecular markers are commonly used in breeding programmes as an easy and inexpensive method for selection interesting genotypes. Number of molecular markers used in marker assisted selection (MAS) in oat is very limited compared to other cereals like wheat or barley. It may be related to the fact that oats is low-input crop. However, there is a few examples of developing molecular markers for important oats traits like resistance to crown rust (Wight *et al.*, 2003; Chong *et al.*, 2004; McCartney *et al.*, 2011), stem rust (Penner *et al.*, 1993), long seed dormancy (Fennimore *et al.*, 1999), dwarf genes (Milach *et al.*, 1997), β -glucan content (Orr and Molnar, 2008) or powdery mildew (Yu and Herrmann, 2006; Okoń and Kowalczyk, 2012a). Yu and Herrmann (2006) developed two molecular markers linked to *Pm5* resistant gene to powdery mildew on common oat, but this gene is not popular in breeding programmes. Okoń and Kowalczyk (2012b) identified molecular marker linked to *Pm6* (OMR2 group) resistant gene, unfortunately this gene is not effective against existed powdery mildew races (Okoń, 2015). In this study, an attempt was made to develop a SCAR marker for *Pm4* resistant gene to powdery mildew in oat. *Pm4* is nowadays the most effective source of resistance against powdery mildew and could be used in breeding programmes to increase the level of resistance of cultivated oat (Okoń, 2015). To developed marker specific to *Pm4* DArTseq methodology was used.

The DArTseq technology results in two sets of markers: dominant and more numerous silico-DArT, and co-dominant and more informative SNP. This high-throughput genotyping method have been used for extending the dense of genetic map (Ren *et al.*, 2015; Milczarski *et al.*, 2016), evolutionary relationships (Al-Beyroutiová *et al.*, 2016), genomic selection (dos Santos *et al.*, 2016) In our study silico-DArT markes were used for identification of molecular markers associated with *Pm4* resistant gene in oat. Number of markers associated with analysed gene was very high. 126 markers showed similar pattern and segregated according to phenotypic observations.

Because of the short sequence of silico-DArT markers, their conversion in a simple PCR method is a very difficult challenge. In available literature there is limited information about successful conversion of silico-DArT markers. Milczarski *et al.* (2016) have found DArTseq markers associated with *Rfc1* gene in rye. Unfortunately the attempt to convert these markers failed. But authors mentioned that sequence data obtained during DArTseq analysis sometimes allow for designation of primers revealing polymorphism within mapping population, but the frequency of such cases is low.

In our study we design primer for 20 potential silico-DArT markers highly associated with *Pm4* gene, among these markers only 5 produce expected patterns in parental line and bulks of resistant and susceptible genotypes. Finally only 3 primer pairs produce expected patterns across the mapping population. It showed that conversion of silicoDArT markers into simple method based on PCR is possible, but efficiency of this process is very low. The available literature indicates that converted DArT-based markers usually maintain the segregation pattern of original DArTs (Ekstein *et al.*, 2014; Shashin *et al.*, 2009). Our investigation showed that a similar situation occurs when we try to convert DArTseq markers. In our study all SCAR markers reveal segregation patterns very similar to the original silicoDArT markers, which confirm correct conversion. Analysis of the correlation of converted silicoDArT markers with the resistance to powdery mildew conditioned by *Pm4* gene demonstrated that they can be useful for selection purposes. It is very important to note that obtained molecular markers are dominant. Thus, additional experiments will be required to design co-dominant marker which will be allow to confirm homozygosity of the genotypes form 'Av1860' \times 'Fuchs' population.

Conclusions

Conducted study is the first to develop effective molecular markers for the *Pm4* powdery mildew resistance gene in oat. They confirm the possibility of converting DArTseq markers into PCR-specific markers, which can be used in marker assisted selection (MAS).

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