Short Communication

Mapping and expression analysis of a Fusarium head blight resistance gene candidate pleiotropic drug resistance 5 (*PDR5*) in wheat

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Abstract
Fusarium head blight (FHB) caused by *Fusarium graminearum* is a serious disease of wheat (*Triticum aestivum* L.), through which grain quality losses are induced by fungal trichotecene mycotoxins such as deoxynivalenol (DON). A class of plasma membrane localized ABC transporter proteins related to the yeast PDR5 (pleiotropic drug resistance5) efflux pump seems to be responsible for partial resistance against trichothecenes in wheat. In order to develop and map a *PDR5*-specific marker linked to Fusarium head blight resistance in wheat, F_3 and F_5 generations obtained from a cross between 'Wangshuibai' and 'Seri82' were used. The analysis of nucleotide sequences of OSPDR5 revealed a high homology to the wheat EST BT009500. Among ten primer pairs developed from this *PDR5*-like EST, one was polymorphic between the parental lines. This PCR-based marker associated with FHB resistance in a 'Wangshuibai' derived mapping population. In this study, Composite Interval Mapping (CIM) analysis detected a QTL in the map interval of *Xm12p17_2-Xpdr5* (consisting of the developed *PDR5*-like gene locus) on chromosome 6BS. This QTL accounted for up to 18% of AUDPC variation. Real-time quantitative analysis showed that wheat *PDR5*-like gene expression was up-regulated during the post-inoculation period of 96 hours in the spike. Our results are in agreement with the hypothesis that the *PDR5*-like gene may be considered as a FHB resistance gene candidate in wheat.

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Fusarium head blight (FHB), a scab principally caused by *Fusarium graminearum* Schw., is a serious disease of wheat, resulting in grain quality losses induced by fungal trichotecene mycotoxins such as deoxynivalenol (DON). The type B trichothecene deoxynivalenol (DON) acts as a potent inhibitor of initiation and termination of eukaryotic protein synthesis (Cundliffe and Davies, 1977). There is strong evidence that during the development of FHB, DON is a virulence factor that enhances disease severity in wheat (Desjardins *et al*., 1996). Manoharan *et al*. (2002) reported that expression of genes such as pleiotropic drug resistance 5 (*PDR5*) could improve resistance to fungal infection, and reduce DON accumulation in wheat cultivars infected with *Fusarium graminearum*. PDR5 is a plasma membrane ABC transporter, which acts as a drug efflux pump (Smart and Fleming, 1996). In yeast, *PDR5* carries two transmembrane and nucleotide binding domains, TMDs and NBDs (Schmitt and Tampe, 2002). Specific amino acids of the nucleotide-binding domains are required for breaking down ATP and releasing the energy necessary for transport (Tutulan-Cunita *et al.,* 2005). The objectives of this research were to develop and map a PDR5-specific marker linked to Fusarium head blight resistance in wheat.

With 'Wangshuibai' a highly resistant Chinese landrace of wheat, *Triticum aestivum* L., and 'Seri82' a

susceptible Mexican spring cultivar (Mardi *et al*., 2005; 2006), 180 F_3 and F_5 lines derived from Wangshuibai/Seri82 cross were selected. Healthy leaves, harvested from the parents and the F_3 individuals, were used for DNA extraction. Total genomic DNA was isolated according to the protocol of Saghai-Maroof *et al*., (1984). Visually symptomatic spikes were counted and Area Under the Disease Progress Curve (AUDPC) was determined for each inoculated spike in F_3 plants and each F_5 derived line as described by Mardi *et al*. (2004, 2005).

The GenBank Nucleotide databases were searched for several monocot [wheat (*Triticum aestivium*), barley (*Hordeum vulgare*), maiz (*Zea mays*)] and Dicot [tomato (*Lycopersicon esculentum*), thale cress (*Arabidopsis thaliana*)] EST's, coding for *PDR5*-like gene. The ESTs having similarity with *PDR5* were used to design oligonucleotide primer pairs using Oligo5 software (www.otset.ed), to assay parental polymorphism. The polymorphic primer pair was used for genotyping of the F_3 individuals. PCR was performed in a total volume of 25 µl, including 1 µl template DNA, 10 pM of each of forward and reverse primers, 3 mM $MgCl_2$, 10 mM dNTPs and 0.2 U Taq DNA polymerase in a 1X reaction buffer (Roche, Germany). The DNA amplification program included an initial 94ºC denaturation for 4 min, followed by 35 cycles of 94ºC for 1 min, 55ºC for 90 s, 72ºC for 3 min, and a final extension of 72ºC for 5 min. PCR fragments were separated on a 1.5% 1X TAE agarose gel stained with Ethidium Bromide and photographed. The purified PCR amplicons were cloned in the pGEM-T Easy vector (Promega, USA). For each polymorphic band, at least eight colonies were picked up and confirmed through PCR, while 5 of them were sequenced with an automatic sequencer (ABI prism ™ 377). Sequences were compared with the database using the BLASTx algorithm (Altschul *et al*., 1997).

The developed *PDR5*-like gene marker was integrated into a linkage map constructed by SSR (simple sequence repeat), AFLP (amplified fragment length polymorphism), RGA (resistance gene analogue) and ESTs (expressed sequence tag sites) (Mardi *et al*., 2005; Naji *et al*., 2008) using the 'group', 'order', 'ripple' and 'try' commands of the computer program MAPMAKER 3.0b (Lander *et al*., 1987). A minimum logarithm of the odds ratio (LOD) score of ten and a maximum genetic distance of 20 cM were used for pairwise linkage analysis. The Kosambi mapping function (Kosambi, 1944) was used to convert recombination frequencies into genetic distances. The QTL analysis and the effect of the developed PCR-based marker for *PDR5* gene was examined by the analysis of variance based on the genotypic and phenotypic data from the F_3 plants, and phenotypic data from the $F_{3:5}$ lines and combined phenotypic data from F_3 and $F_{3:5}$ with the SAS/STAT software (SAS Institute Inc. 1990). Interval mapping was conducted using PLABQTL software (Utz and Melchinger, 1996).

A single central floret of the spikelet was inoculated with 10 µl of a macro-conidial suspension (100 000 conidia m/l) of *Fusarium graminearum*. To provide high humidity, the infected heads were covered with a transparent plastic shelter after inoculation, as described by Mardi *et al*., (2005). After 20 hours, the covers were removed. All plants were incubated in a humidity chamber set at 21-22ºC for 18 h of light and 6 h of darkness. Control plants of the same parental genotypes were sprayed with water containing 0.2 percent Tween 20, and incubated in the same conditions. The whole wheat spike was harvested at 0, 24, 48 and 96 h post-inoculation. Total RNA was extracted from inoculated and control spikes using the RNeasyplant mini kit (Qiagen), according to manufacturer's instructions. RNA concentration and integrity was checked with a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). Only RNA samples with 260/280 wavelength ratio between 1.9 and 2.1 and 260/230 wavelength ratio greater than 2.0 were used for cDNA synthesis. The quality of RNA samples was also assessed by electrophoresis on 1% formaldehyde agarose gels. For real-time quantitative PCR (qRT-PCR), 1 µg total RNA was treated with 1U amplification grad DNaseI (Qiagen), then reverse transcribed using I Script cDNA synthesis Kit (Bio-RAD, USA), according to manufacturer's instruction. The resulting cDNA was diluted 1:10 with water. Gene specific primers were designed using Beacon Designer soft-

Table 1. Two primer pairs designed from wheat PDR5 gene for quantitative real-time PCR.

Primer	Forward sequence (5´-3´)	Reverse sequence (5´-3´)	Annealing Temp.(ºC)
18s	ATCACCGTATCAGGCTATCC	GTCCTCATCACAATTGCAGC	58
PDRt	GCACGTATATCGGGATACTC	CTAGAGTACTGTGTAGGG	58

ware version 4 (Premier Biosoft International, USA). PCR was conducted in a total volume of 25 µl with a final concentration of 1x SYBR Green PCR master concentration (Bio-RAD, USA), 6 µl cDNA and 10 pM of each forward and reverse gene-specific primer (Table 1). Cycling conditions consisted of a denaturation step at 95ºC for 5 minutes, followed by 45 cycles at 95ºC for 1 minute, 58ºC as annealing temperature for optimized primers for 90 seconds, and 72ºC for 2 minutes. After the amplification, a melting step of 90 cycles of slow temperature rise from 55ºC to 95ºC at the rate 0.5ºC/10 s was carried out. Melting curve analysis was performed after each RT-PCR run to verify the specificity of SYBR Green dye, and the absence of primer-dimers. The wheat 18s ribosomal RNA gene was used as an internal control. The ΔΔCT method of relative gene quantification (Applied Biosystem, 1997) was used to calculate the expression level of the whole spike and floret organs.

Throughout a nucleotide collection (nr/nt) blast search with maximum target sequences of 100 and expected threshold 10 (match/mismatch 1,-2) using the *PDR5* sequences from dicots and monocots, one EST was retrieved from the wheat genome EST database (accession no. BT009500) having 85% homology with the rice putative *PDR5*-like gene (accession no. AY332479). Among the set of primers tested, one from the non-conserved region of the gene (PD5) generated a polymorphic band (Fig. 1). The sequence of the polymorphic band, shown in Figure 2, was verified on GenBank with the original BLAST alignment, and showed 90% homology with *Oryza sativa* gene for PDR-like ABC transporter (accession no. AJ535047). An analysis of variance using general linear model (GLM) revealed a highly significant negative influence of the developed PCR-based marker on AUDPC in populations derived from Wangshuibai/Seri82 (Table 2). The developed marker was integrated into a linkage map including 21 AFLP and 3 SSR loci covering a genetic distance of 211.2 cM, and providing a partial linkage group for chromosome 6B. Composite interval mapping (CIM) analysis detected one QTL mapping to chromosome 6BS for AUDPC. A QTL in the map interval *Xm12p17_2-Xpdr5* on chromosome 6BS was detected in both generations and accounted for up to 18% of AUDPC phenotypic variation. Real-

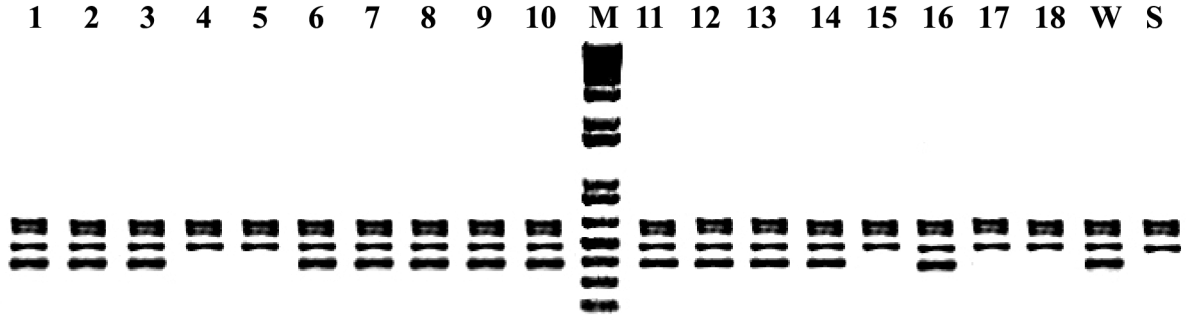

Figure 1. The PCR products amplified by the designed primer pair PD5 showed polymorphism between the parental lines and the F_3 individuals. M, S and W are the molecular size marker (1 Kb plus DNA ladder, Invotrogen, INC, USA), 'Seri82' and 'Wangshuibai', respectively.

ATGGGATCAATGTATGCTGCGTGGAAATTGTCGGTACCTCAGAAGATGAAAATCATGGCACCTTGCCAA-
CAACGCAATGTAAACAGAACAATGCAATGTAAAAATTTCAGGCTTGGAAGGTAGTTGCAGGCGCCTTGC-
CAACAACGCAATGTAAAAATTTCAGACATATTTCCACGCGCTCGACCTGTCCTTTATGTCGTGTGGAAGAG-
GAGGGTTCCTTTCATGCGTTAGTTACATGCACTAACGCGCATCTGATTTGGTTGAATATGAGAACTAGGTG-
GCCTCTCCCCTCTAATGAAATTCTCATTGACAATGGCAAGGATTGGTTACTGCATGTGCTGGTTAACTG-
TACGGATGATATCCGGGATATGGTGATTATGGTAGTCTGGCGAATTTGGCAATTGCGGACATATCAATCA-
CATGGCAAGGAAATCCCACCAACGGATGTCACGGTGGAGTTCCTCGATAGCTACTATAGGTCCAT-
CAAACTTGCAGGTCGGTTTAGCATGGAGGAAATAATCAAGGGCAAGATGACAGTGTCTGCCGAGT-
GCTCAGGTTCTTTCGACAAAGTGAAGCCCCCGACCTAAACTTCCTA

Figure 2. Sequence of the polymorphic band amplified by the designed primer pair PD5 on the non-conserved region of Bt009500, isolated from 'Wangshuibai'. Forward and reverse primers are shown with arrows.

Table 2. The map intervals, chromosomal locations, logarithm of odds (LOD), the percentage of explained phenotypic variance (VE) and effects of quantitative trait loci (QTL) detected for area under the disease progress curve (AUDPC) using F_3 and $F_{3:5}$ populations derived from a Wangshuibai/Seri82 cross. QTL analysis was carried out by composite interval mapping (CIM).

Generations	Map interval	Chr.	LOD	VE	Effect[1]
F_3	Xm12p17_2-Xpdr5	6BS	7.67	18.0	-2.615
F_5	Xm12p17_2-Xpdr5	6BS	3.88	9.6	-0.891
Combined analysis	Xm12p17_2-Xpdr5	6BS	1.70	4.3	-0.756

[1] Negative effects indicate the direction of the QTL response on AUDPC for alleles contributed by 'Wangshuibai'.

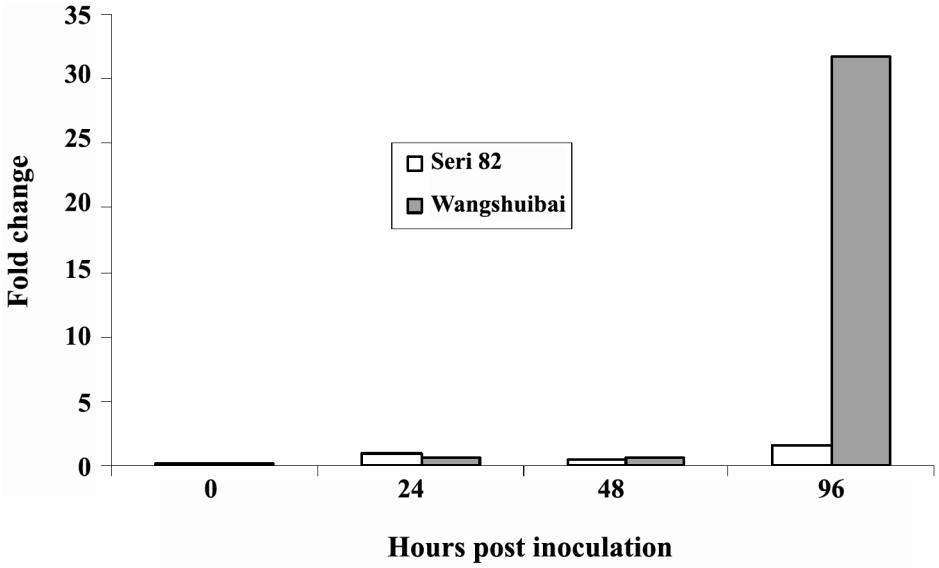

Figure 3. Real-time analysis of wheat *PDR5* gene in 'Wangshuibai' and 'Seri82' at 0, 24, 48 and 96 h post-inoculation for the whole *Fusarium graminearum* spike inoculated.

time quantitative analysis showed that wheat *PDR5*-like gene expression was up-regulated during a post-inoculation period of 96 hours in the spike (Fig. 3).

In this study, the primer pairs designed based on the conserved sequence amplified similar fragments in size between two resistant and susceptible parents. This was expectable, as *PDR5* belongs to a multigene family with highly conserved sequence, such as nucleotide binding fold (Theodoulou, 2000). Mitterbauer and Adam (2002) reported that the PDR gene exists in plant genomes as a large gene family with highly conserved sequence. Van den Brule and Smart (2002) indicated about 15 *PDR5* genes within the genome of *Arabidopsis thaliana*. Jasinski *et al.* (2003) predicated fifteen PDR genes in rice genome. Common wheat is a hexaploid plant, consisting of A, B and D genomes, and may contain up to 75 PDR genes (Mitterbauer *et al*., 2003).

The designed primer pairs based on the non-conserved sequence amplified a polymorphic fragment in resistant parent 'Wangshuibai'. Sequence analysis of the polymorphic band showed one intron with flanking primers at the ends of the sequence. The developed PCR-based marker with a significant negative effect on AUDPC variation was integrated into partial linkage groups on the short arm of 6BS chromosome, where Lin *et al*. (2004) detected FHB resistance QTL, *Qfhs.ndus-6BS*, derived from 'Wangshuibai'. This is the first report on the detection of a QTL in 6BS chromosome, co-segregated with a FHB gene candidate. Five *PDR*-derived EST were mapped on 6B chromosome in wheat through comparative genomics between Rice, Maize and *Arabidopsis* (http://wheat.pw.usda.gov/cgi-bin/west-sql/bin_candidates.cgi?bin=6BL5-0.40-1.00). The over-expression of *PDR5*-like gene may describe the involvement of this gene with resistance to FHB in

'Wangshubai,' yet this needs to be confirmed. Characterization of this wheat *PDR5*-like gene homologue seems to be a straightforward strategy to support the suggestion that the *PDR5* be considered as a FHB resistance gene in wheat.

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