Journal of Plant Biochemistry & Physiology
ISSN: 2329-9029

Jawhar et al., J Plant Biochem Physiol 2018, 6:4
DOI: 10.4172/2329-9029.1000225

Research Article Open Access

EcoTILLING in *Cochliobolus sativus* Isolates Reveals Polymorphisms in the XYL1 and XYL2

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Abstract

The fungus *Cochliobolus sativus* is a cereal pathogen that in addition to its role in plant disease has potential industrial applications. It has been shown to be an efficient producer of xylanase. The enzyme breaks down hemicellulose and therefore identification of isolates with high xylanase activity is one target for enhancing biofuel production. Genetic variants of XYL1 and XYL2 genes were found to be associated with altered xylanase activity. However, although these two major genes have been described, relatively little is known about the patterns of xylanase gene diversity in this pathogen. In this work, we have developed a low-cost EcoTILLING method for single-nucleotide polymorphism (SNP) and small indel discovery in XYL1 and XYL2 genes from fungal isolates that is suitable for most laboratories. The approach utilizes self-extracted single-strand-specific nucleases and standard agarose gel electrophoresis for polymorphism discovery. Gene-specific primer pairs were used to optimize enzymatic mismatch cleavage and polymorphism discovery on a panel of 56 *C. sativus* isolates. Based on the nucleotide polymorphisms detected, these isolates were organized into four haplotype groups. These haplotype groups were found to correlate with differences in xylanase production. Seven novel nucleotide variation was confirmed by sequencing. The discovered polymorphisms serve as genome specific markers for screening of additional *C. sativus* isolates. The ability of rapid and low-cost genotypic methods to categorize xylanase production may be of value in both basic research and industrial settings.

Keywords: EcoTILLING; Low-cost polymorphism discovery; Xylanase; Single-strand specific nuclease XYL1, XYL2; *Cochliobolus sativus*

Introduction

Xylanase (endo-1, 4-B-xylanases, EC 3.2.1.8) is a class of enzymes that are involved in the breaking down of hemicellulose. Nowadays, it has attracted special attention due to its potential applications in many processing industries [1]. In nature, plant pathogens use these and other enzymes to degrade plant cell walls. As such, characterization of xylanases and xylanase gene diversity has implications for plant-pathogen interaction and disease control [2-4]. The ability to break down plant cell walls also has important applications for human endeavours such as in the paper making industry and more recently for the production of biofuels [5]. Although xylanases from eubacteria and archaebacteria have considerably higher temperature optima and stability than those of fungi, the amount of enzyme produced by these bacteria is comparatively lower than that produced by fungi [6-8].

Fungal plant pathogens are considered promising sources of cell wall-degrading enzymes [7-9]. The haploid fungus *Cochliobolus sativus* (anamorph *Bipolaris sorokiniana*), a necrotrophic cereal pathogen, has been shown to produce cell wall degrading enzymes (CWDEs) including xylanase [10]. Xylanases are likely to be particularly important CWDEs in interactions between *C. sativus* and barley plants [11]. Different types of xylanase genes XYL1 and XYL2 have been characterized in *C. sativus* [12]. which are code for typical members of glycoside (glycosyl) hydrolase family 11 [13].

Detecting variations at single nucleotide positions and small nucleotide insertions or deletions are considered to be the most common forms of genetic diversity in natural populations [8,11]. Haploytpe blocks of genetic variation caused by linkage disequilibrium can be detected in different species. While the nucleotide variation causative for observed phenotypic variation may be difficult to demonstrate experimentally, the genotype codes for the phenotype is a useful tool in the plant, animal, microbe, and medical sciences [14-17]. Substantially, identification of related haplotype blocks can serve to understanding of causative genetic variation.

EcoTILLING approach for rapid discovery of DNA polymorphisms has been widely used for evaluating plant genomes, and to a lesser extent in humans and animals [18]. It was an adaptation of the of TILLING (Targeting Induced Local Lesions IN Genomes) technique, allows high-throughput analyses of natural genetic diversity in populations [18,19]. In this method, a single-strand specific endonuclease extracted from celery (CEL I) is used to cleave DNA where there are mismatched bases in the DNA heteroduplexes of PCR products. Cleaved fragments observed by gel electrophoresis indicate the presence of nucleotide variations. Because cleavage reveals only heterozygous polymorphisms, a sample pooling strategy involving at least one reference DNA and one test DNA is commonly employed as a means of uncovering homozygous differences between two samples [20]. Experiments indicated that EcoTILLING is highly efficient for detecting genetic variations associated with target traits for crop improvement. Nieto et al. [21] found one haplotype consisting several grouped SNPs controlling virus susceptibility in a natural melon population. Many SNPs in a dozen genes were also identified in wild populations of *Populus trichocarpa* [22] and in the Musa gene pool [23]. Key SNPs in the brassica FAE1 gene were also detected and these may be exploited for finding new low erucic acid sources [24].

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Received October 31, 2018; **Accepted** November 15, 2018; **Published** November 25, 2018

Citation: Jawhar M, Till BJ, Albaterni A, Skiheita A, Arabi MIE, et al. (2018) EcoTILLING in *Cochliobolus sativus* Isolates Reveals Polymorphisms in the XYL1 and XYL2. J Plant Biochem Physiol 6: 225. doi: 10.4172/2329-9029.1000225

EcoTILLING involves PCR amplification of target gene regions and create heteroduplexed molecules where polymorphisms exist in the amplified DNA. Heteroduplexes are subjected to cleavage by incubation with single-strand-specific nucleases, and cleaved fragments could be observed by gel electrophoresis to indicate SNP and small indel variations. Since cleavage reveals only heterozygous polymorphisms, a sample pooling strategy involving at least one reference DNA and one test DNA is commonly employed as a means of uncovering homozygous differences between two samples [20]. With this method it is possible to group samples according to haplotype banding pattern. Identification of the nucleotide variation requires sequencing of only one member of each haplotype group, that can reduce costs of the assay. While many works have employed fluorescence detection to improve sensitivity and denaturing PAGE for base-pair resolution, lower cost methods can be employed. Single-strand-specific nucleases such as CEL I that nick a single mismatched region in the heteroduplex molecules, will eventually cleave both strands at the mismatch resulting in a double strand break that can be identified using conventional native agarose gel electrophoresis [25-27]. In addition to a low-cost readout platform, CEL I can be self-extracted from celery or mung beans making the entire procedure low-cost [28].

In the present study we established EcoTILLING in *C. sativus* to survey a panel of isolates for allelic variants in candidate genes implicated in regulating xylanase production. As a proof of principle, Jawhar et al. [29] developed this method for barley spot blotch pathogen (*C. sativus*) to survey a panel of 50 isolates for allelic variants in different candidate genes. This panel was previously phenotyped for variation in the production of xylanase [30]. We chose XYL1 and XYL2 as candidate genes because of their known functions in xylanase production in *C. sativus*. A correlation between haplotypes and xylanase activity was observed. This work shows the utility of a low-cost EcoTILLING strategy using self-extracted nuclease and agarose gel electrophoresis for detecting natural nucleotide variation in the xylanase genes of *C. sativus* isolates. Based on the wide use of EcoTILLING in plant genomes and the establishment of the method for *C. sativus,* we envision broad applicability of this approach for many fungi.

Materials and Methods

Fungal isolates

During 20 years, isolates of *C. sativus* were obtained from leaves of barley and wheat showing spot blotch symptoms. In previous works [31], 56 single spore isolates were isolated from a single colony under the microscope using a sterile glass needle. Each colony was then eventually transferred to Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI. USA) with 13 mg/l kanamycin sulphate added after autoclaving, and incubated for 10 days, at 22 ± 1°C in the dark to allow mycelial growth and sporulation.

Xylanase production

A natural population of 56 *C. sativus* monosporic isolates selected on the basis of morphological, physiological and xylanase production criteria [29-31]. were used in this study. Xylanase was produced by submerged culture as described by Bailey et al. [32] using 1% birchwood xylan as the substrate. The xylan solution and the enzyme at appropriate dilution were incubated at 55°C for 5 minutes and the reducing sugars were determined by the dinitrosalicylic acid procedure [33], with xylose as the standard. The released xylose was measured spectrophotometrically at 540 nm. One unit (U) of xylanase activity was determined as the amount of enzyme releasing 1 µmol xylose/ml per minute under experimental conditions.

DNA extraction and primers design

Genomic DNAs were prepared according to standard protocol [34]. DNAs were evaluated for quality and quantity using a standard agarose gel assay as previously described [35]. Each sample was normalized to a concentration of 20 ng/µl in TE buffer. Sequences for XYL1 and XYL2 of *C. sativus* were downloaded from GenBank (Accession numbers AJ29724 and AJ303047, respectively) and aligned with each other and PCR primers specific for XYL1 or XYL2 were designed based on the genome sequence of XYL1 and XYL2 genes in *C. sativus* ND90 (GenBank:M2R232; Table 1) [35].

PCR amplification and heteroduplex formation

In a preliminary experiment, the genomic DNA from five to eight isolates was pooled and then the isolates evaluated separately in order to determine nucleotide variation between tested samples. The PCR reaction was performed according to Jawhar et al. [29] in a 20 µL reaction volume containing 1U AmpliTaq® DNA polymerase, 60 µM dNTPs, 75 ng of genomic DNA, and 1.0 µM primers in 1X PCR Buffer (1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris•HCl, pH 8.3 at RT). After an initial denaturing step at 94°C for 4 min, the DNA was amplified through 20 cycles consisting of denaturing at 94°C for 5 sec, annealing at 65°C for 1 min decreasing by 0.5°C per cycle, and extension at 72°C for 1 min. The samples were then subjected to an additional 30 cycles consisting of denaturing at 94°C for 5 sec, annealing at 55°C for 1 min, and extension at 72°C for 1 min, with a final extension of 5 min at 72°C. Denaturing and re-annealing step were included at the end of the PCR reaction (99°C for 10 min, followed by 70 cycles of 70°C for 20 s decreasing by 0.3°C per cycle) to allow the formation of heteroduplexes.

CELI digestion of the heteroduplexes and agarose gel electrophoresis detection

CELI digestion was carried out according to Till et al. [28] with some modifications. The formed heteroduplexes (20 µL) were mixed with 20 µL nuclease mixture, containing 6 µL CJE Buffer (10 mM Hepes, pH 7.5, 10 mM $MgSO_4$, 10 mM KCL, 0.002% Triton X-100, and 0.2 µg/mL BSA); 2.4 µL crude celery juice extract (CJE) preparation, and 11.6 µL ultrapure water. The digestion was carried out at 45°C for 45 min followed by incubation on ice to stop the reaction, and 4 µL of 10 × gel loading buffer was added to each sample. The digested products were separated on 2% agarose gels with 1 × TAE buffer at 110 V for 50 min, stained in an ethidium bromide buffer for 15 min, and then visualized by Gel Doc XR (BioRad Laboratories, Inc.).

Genotyping and verification by sequencing

Differences among the digested fragments from various DNA pools were assessed visually by recording molecular weights of bands representing cleavage products. Isolates had the same band patterns were considered as the same genotype for haplotype grouping. The cleaved bands were purified using an Ultrafree-DA DNA extraction kit (Millipore, Bedford, MA, USA) and sequenced by the dye terminator method on an Analyzer (ABI 310, Perklin-elmer, Applied Biosystems, USA). The sequences were used to validate nucleotide polymorphisms and haplotype associations determined by EcoTILLING. Geneious Pro Software v 8.0 (Biomatters Ltd; http://www.geneious.com) [36] was applied for detecting SNPs between sample data and a reference sequence.

Results and Discussion

A *C. sativus* EcoTILLING strategy was developed for the evaluation of nucleotide diversity in xylanase genes (Figure 1). A key agent for precise polymorphism discovery was the development of gene-specific primers. However, in our study, three of the nine designed primer pairs could amplified a single gene target (Table 1), and each unique fragment visible after enzymatic digestion and agarose gel electrophoresis was scored as a SNP. Previous works with human and polyploid bananas shows that false positive and negative error rates are approximately 5% [19,23]. Across the three amplicons a total of 7 different NPs were identified among the 56 isolates (Figure 2). The remaining primer combinations were not suitable for EcoTILLING due to miss-priming and single primer amplification as revealed in the primer testing experiments (data not shown).

Band patterns of different pools, the 56 *C. sativus* isolates were divided into four groups, namely, haplotype A, B, C and D (Figure 2). The major variations in band patterns were only considered for haplotype classification, whereas those with minor differences were disregarded. The obtained sequences consistently demonstrated target fragment sequences in the two genes of the same haplotype group, indicating that a relatively accurate SNP genotyping could be achieved in the target genes via the EcoTILLING technique using agarose gel electrophoresis.

Samples from each haplotype were subjected to Sanger sequencing to confirm the EcoTILLING data (Table 2). While checking the correlation between the haplotype detected by EcoTILLING and xylanase phenotype of those *C. sativus* isolates, four haplotypes can be observed (Figure 2; Table 3). This suggested that the haplotype based on band patterns of EcoTILLING analysis with agarose gel detection could well reflect the variation of the xylanase genes among *C. sativus* isolates.

Enzymatic mismatch cleavage was previously shown to be highly sensitive allowing for the efficient discovery of heterozygous mutations in samples pooled up to eightfold [37]. We utilized this assay sensitivity to develop a strategy for the identification of all nucleotide polymorphisms in a haploid genome type by pooling samples prior to screening. Together, this allows for hypothesis testing of the evolutionary origin of nucleotide polymorphisms. To take advantage of agarose gel detection, special attention must be given to the quality and quantity of PCR products. First, it is important that a single specific product is obtained from a PCR reaction since the presence of non-specific products may lead to heteroduplex formation. Non-specific amplification would result in the detection of CEL I-cleaved products even in the control samples. Therefore, it is important that once a SNP is detected in a pool, the individuals of the pools be tested separately to confirm that the cleaved products are a result of heteroduplexes formed across members of the pool and not a result of non-specific amplification. Secondly, it is critical to have a high yield of the amplicon. Products at concentrations less than 5 ng/µl would not be detected on regular agarose gels.

The polymorphisms found for XYL1 and XYL2 readily revealed the SNPs/indels associated with differences in xylanase contents [38,39]. Thus, our results are consistent with other studies showing that SNPs/indels in xylanase corresponded to loss of function of these genes in *C. sativus* [40,41]. Therefore, detecting new *C. sativus* isolates with high xylanase content genetic background in this study using EcoTILLING would be a very efficient method for augmenting xylanase genetic resources.

Differences of EcoTILLING patterns among the different isolates were easy to detect by agarose gel electrophoresis, which reflect the highest genetic diversity for the investigated genes in *C. sativus*. Our results are in agreement with those obtained by Arabi and Jawhar [31] who reported high genetic diversity among Syrian *C. sativus* isolates. Zhong and Steffenson [42] documented that the diversity among this pathogen isolates may be due to their deriving from the same source population or transporting from area to another by their hosts.

On the other hand, the EcoTILLING used in this study, compares favorably to the most common alternative–full sequencing. For polymorphism discovery in populations of haploid such fungi, the frequency of haplotypes will determine which of the most advantages, and longer ratios of haplotypes to sampled isolates favor sequencing. With many fewer haplotype than isolates, EcoTILLING greatly reduces the number of sequences that needs to be determined. Further, this method is several fold cheap than full sequencing.

This study demonstrates that low-cost EcoTILLING based on self-extracted nucleases and agarose gel electrophoresis has been successfully adapted for detecting natural polymorphisms in XYL1 and XYL2 genes *C. sativus* population. These polymorphisms genes allowed grouping of fungi isolated from barley leaves into four haplotypes, which were associated with different xylanase production. EcoTILLING is shown to be a fast, convenient, and effective method for detecting SNPs in genes of haploid species, such as fungus. The method has been widely adapted for many crop species [43-45]. Having established EcoTILLING for *C. sativus*, we expect that it can be similarly adapted for many microorganisms. Moreover, this accurate and robust screening and molecular diagnostic of fungal isolates with high enzymatic activity will be essential for development of applications of industrial enzymes, that can transform agriculture and healthcare, use renewable resources to bring greater efficiency into industrial processes, check environmental degradation and deliver a more bio-based economy.

Primer name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Xyla	CCCCAGTATCATTTCATCCTGCGATTC	TTGCGCCATCATCATGCTAGCTACTTC
Xylb	CCCCAGTATCATTTCATCCTGCGATTC	AAGTGCTATTGCGCCATCATCATGCTA
Xylc	CCCAGTATCATTTCATCCTGCGATTCA	TTGCGCCATCATCATGCTAGCTACTTC
Xyld	TTTCATCCTGCGATTCAGGCTCAACTA	AAGTGCTATTGCGCCATCATCATGCTA
Xyle	CCCAGTATCATTTCATCCTGCGATTCA	AAGTGCTATTGCGCCATCATCATGCTA
Xylf	CCCCAGTATCATTTCATCCTGCGATTC	AAGTGCTATTGCGCCATCATCATGCTA
Xylg	CCCAGTATCATTTCATCCTGCGATTCA	TTGCGCCATCATCATGCTAGCTACTTC
Xylh	TTTCATCCTGCGATTCAGGCTCAACTA	AAGTGCTATTGCGCCATCATCATGCTA
Xyli	CCCAGTATCATTTCATCCTGCGATTCA	AAGTGCTATTGCGCCATCATCATGCTA

Table 1: EcoTILLING primers designed for this study.

Gene	Position	Reference	Haplotype			
		Allele	A	B	C	D
XYL1	244	A	T	A	A	T
	257	G	G	C	G	G
	295	CA	CA	GT	G	T
	301	A	A	T	T	A
	324	T	T	A	T	T
XYL2	411	C	C	T	C	G
	460	G	G	C	T	C

Table 2: Sequence comparison for target regions of the XYL1 and XYL2 genes between haplotypes (A, B, C and D) and reference *C. sativus* isolate ND908.

Isolate	Xylanase (U/g)	Haplotype	Isolate	Xylanase (U/g)	Haplotype
Cs61	73	D	Cs16	1100	A
Cs64	73	D	Cs31	1200	A
Cs30	75	D	Cs51	1200	A
Cs56	77	D	Cs8	1253	A
Cs22	129	D	Cs37	2630	C
Cs58	130	D	Cs39	2700	C
Csl1	141	D	Cs42	2720	C
Cs56	170	D	Cs69	2800	C
Cs20	180	D	Cs41	2900	C
Cs59	180	D	Cs54	2900	C
Cs23	188	D	Cs5	2950	C
Cs33	188	D	Cs53	2950	C
Cs17	202	D	Cs6	3398	C
Cs57	203	D	Cs9	3900	C
Cs24	206	D	Csl	4095	B
Cs34	206	D	Cs45	4200	B
Cs62	208	D	Cs40	4900	B
Cs21	213	D	Cs48	5330	B
Cs16	293	D	Cs65	5400	B
Cs28	298	D	Cs2	5403	B
Cs32	300	D	Cs38	5710	B
Cs13	302	D	Cs52	5750	B
Cs70	306	D	Cs7	5824	B
Cs15	322	D	Cs14	6130	B
Cs14	361	D	Cs!	6171	B
Cs29	372	D	Cs55	6300	B
Cs3	408	D	Cs10	6365	B
Cs12	1099	A	Cs50	6610	B
LSD (-0.05)					

Table 3: Association of xylanase production in *C. sativus* isolates, with haplotype groupings.

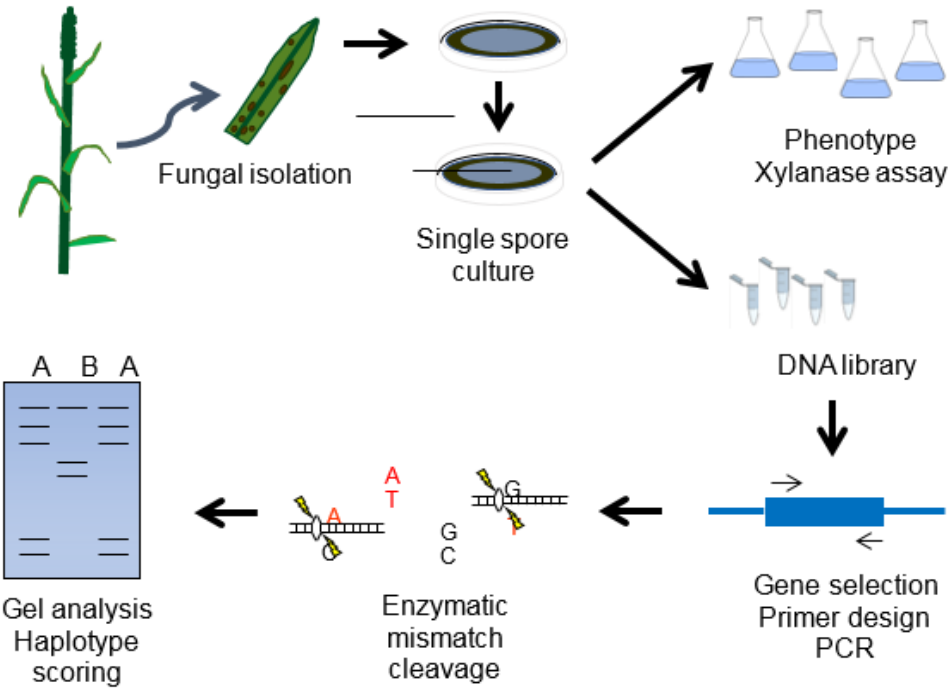

Figure 1: Scheme for EcoTILLING *Cochliobolus sativus* isolated from barley leaves. Disease spots are isolated and passed through a single spore culture. Samples are phenotypically evaluated for xylanase activity and DNA prepared from each culture. Gene-specific primers are designed to target loci. PCR is performed followed by a denaturing and annealing step to create heteroduplexed amplicons in samples where polymorphisms exist. Amplicons are subjected to enzymatic mismatch cleavage and cleavage products visualized by agarose gel electrophoresis. Samples with the same banding pattern represent the same haplotype grouping.

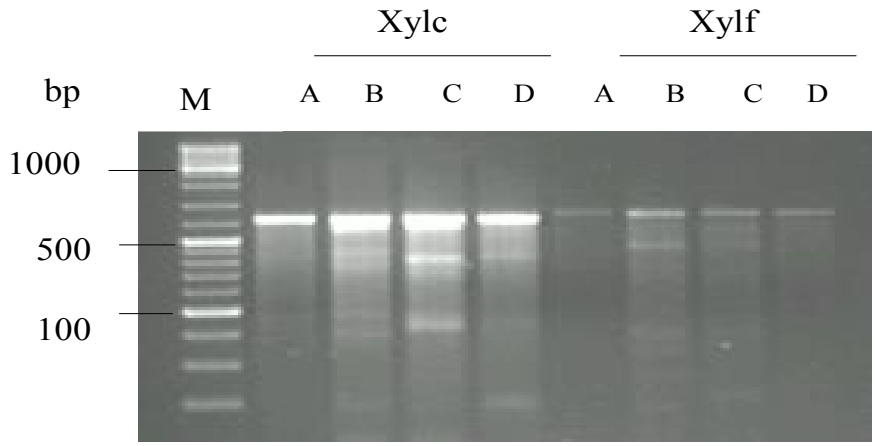

Figure 2: Enzymatic mismatch cleavage band patterns (A to D) in pools of *C. sativus* for testing primer pair Xylc and Xylf. M: molecular weight marker (HinfI; MBI Fermentas, York, UK).

Acknowledgments

The authors would like to thank the Director General of AECS and the Head of Molecular Biology and Biotechnology Department for their support. They would also like to thank the Food and Agriculture Organization of the United Nations and the International Atomic Energy Agency through their Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture for supporting the laboratory experiments.

Conflict of Interest

The authors declare that they have no conflicts of interest to this work.

Ethical Statements

The authors declare that this work has not been published in whole or in part elsewhere; and that appropriate attribution and citation is given for any material reproduced from any other source including the authors' prior publications.

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