IDENTIFICATION OF THE WRKY TRANSCRIPTION FACTORS IN *AGARICUS BISPORUS* (WHITE BUTTON MUSHROOM)

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ABSTRACT

With the changing environmental conditions, organisms can reprogram their transcriptome through transcription factors. WRKY transcription factors are a class of sequence-specific DNA binding transcription factors found almost exclusively in plants and are key regulators of gene expressions. So far, only two WRKY homologues have been identified from non-plant species, *Giardia lamblia* and *Dictyostelium discoideum*. Some WRKY proteins exist as chimeric proteins combining NBS-LRR (nucleotide binding site - leucine rich repeat) proteins and WRKY domains. During the study, WRKY domain was searched in silico in the genome sequence of *Agaricus bisporus* and also the priming sites were determined. Two WRKY and one Nucleotide Binding Site (NBS) primers amplified the WRKY domains in white button mushroom. The sequences of the amplicons were BLAST compared against *A. bisporus* genome. Surprisingly, *Agaricus* genome showed the presence of WRKY domain at multiple sites and also different type of WRKY domains could be identified in the genome. Also the NBS (associated with WRKY domain) primer for disease resistance gene amplified fragment in the *A. bisporus* showing the presence of disease resistance genes in the genome. This is a first report of presence of WRKY domain (specific to plants) in *A. bisporus* genome. Characterization of WRKY domain in *A. bisporus* is under progress.

INTRODUCTION

To cope with variable environmental conditions, organisms have evolved a great capacity to extensively reprogram their transcriptome in a highly dynamic and temporal manner through an integrated network of transcription factors. The WRKY transcription factors are a group of regulatory proteins predominantly involved in stress responses [1]. Together with other transcriptional regulators, WRKY proteins enable plants to better adapt to the changing environment and respond properly to internal and external stimuli. WRKY factors are key regulators, both positive and negative, of both biotic and abiotic stresses, seed development, seed dormancy and germination, development and senescence. The WRKY protein family contains a highly conserved motif spanning about 60 amino acids in all the family members [2]. Within this domain, there is an almost invariable heptapeptide signature WRKYGQK at the N-terminus and a novel Zincfinger-like structure at the C-terminus. The WRKYGQK is the most dominant form of the signature followed by WRKYGKK and WRKYGEK [2, 3), however, there are at least 35 variants of this motif present in plant and non-plant species. The WRKY domain can be characterized as WRRY, WSKY, WKRY, WVKY, or WKKY. WRKY proteins preferably bind to the consensus sequence TTGACC/T, the so-called W-box, which is usually enriched in the promoter region of WRKY target genes such as stress responsive genes.

Members of the WRKY family can be classified into three groups according to the number of WRKY domains and the pattern of the Zinc finger motif in the WRKY protein [2]. Generally, group-I WRKY transcription factors contain two WRKY domains with distinct functions. Previous studies have demonstrated that the C-terminal WRKY domain mediates sequence specific binding to the target DNA [4, 5, 6]. It has been proposed that the N-terminal WRKY domain increases the affinity or specificity of these proteins to the target sites. Group II and III WRKY transcription factors contain one WRKY domain with a C_2H_2 zinc finger motif and C_2H_XC zinc finger motif. Based on a phylogenetic analysis of the WRKY family, the members of group II can be divided into five subgroups: IIa, IIb, IIc, IId, and IIe [2].

Since the cloning of a WRKY gene cDNA from *Ipomoea batatas* [6], a large number of WRKY protein genes have been cloned from different plant species [7, 8, 9]. So far, only two WRKY homologues have been identified from non-plant species, *Giardia lamblia* [10] and *Dictyostelium discoideum* [11]. Some WRKY proteins exist as chimeric proteins combining NBS-LRR (nucleotide binding site - leucine rich repeat) proteins and WRKY domains [12, 13, 14].

The present work intended to study the presence of WRKY transcription factors in Macro-fungi and their role in stress response in the mushroom.

MATERIAL AND METHODS

Database search

WRKY gene sequences were obtained by multiple BLAST searches of NCBI database (http://www.ncbi.nlm.nih.gov) using the WRKY domain sequences. The NBS sites related to WRKY transcription factors were also identified and sequences were obtained from the database. The whole genome sequence of *Agaricus bisporus* was obtained from NCBI database and the whole genome was scanned for the presence of WRKY protein domain and their nucleotide binding sites. Primers were designed for the amplification of the WRKY factors and tested in silico for their success using Genious 6.0.

DNA isolation

Genomic DNA was extracted from the fruit body of *A. bisporus* following standard cetyl trimethyl ammonium bromide (CTAB) isolation protocol. The LN2 frozen fruit body was ground with mortar and pestle to powder form. Lysis was done using preheated CTAB buffer (2% CTAB, 20 mM EDTA, 100 mM Tris-Cl at pH: 8.0, 1.4 M NaCl, and 0.2% marcaptoethanol) @ 5ml per gram tissue followed by chloroform/iso-amyl alcohol (24:1) extraction twice. The supernatant was taken after centrifugation and DNA was precipitated with 0.66 volume of cold isopropanol, collected by centrifugation or spooled out. DNA was washed and air dried briefly and 250-500 µl of TE was added and left overnight before adding 1 µl (10ng/ml) RNase to each 1 ml TE/DNA mixture and incubating for 45 minutes at 37ÚC. DNA was spooled out, air dried and re-suspended in 0.5 to 1 ml T.E. (8-24 hours; final concentration c. 0.1 to 1 µg/ul) and stored frozen at -20 ÚC.

PCR amplification of WRKY transcription factors

Two WRKY primers pairs i.e. A7G1F + A7G1R and W3 F + W3R along with One NBS primer pair M-13R1 + M1495R were used to amplify WRKY domain in *A. bisporus*. Out of the three primer pair identified in silico to have binding efficiency with WRKY domains in *A. bisporus* genome, two primer pair successfully amplified the WRKY domains. The sequences of the primers are: WRKY Primer sequences

A7G1F – 5' CCTTCTCCTTCCCTTCGACT 3'; A7G1R – 5' AATGATCTCGGTGAGGTCAGA 3'

Leucine rich repeat nucleotide binding site primers

M13R1F- 5' CGGCCAAGTCGTGCAAYVAKRTCRTGCA 3'; M131495R – 5 ' YTTNARNGCNARNGGNARNCC 3'

The successful primer pairs were tested in 6 strains, 3 fertile and 3 non-fertile single spore isolates of *A. bisporus*. PCR reactions were carried out in 15 µl. The mixture contained 1 µl $MgCl_2$ (25mM); 1.5 µl of 10X PCR Buffer; 1.2 µl 10mM dNTP mix; 0.2 µl 5U Taq DNA polymerase; 1 µl of 10 pM each primer and 50ng template DNA. The PCR conditions were denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 30s; annealing at 56°C for 30s; elongation at 72°C for 1 min. The final elongation at 72°C is done for 10 min.

Elution, sequencing and analysis of amplified WRKY fragment

The amplified fragments were eluted from the gel using Qiagen gel extraction kit and were sequenced. The sequences obtained were subjected to BLAST against *A. bisporus* genome sequences in NCBI database. The matching sequences were downloaded and subjected to phylogenetic analysis using MEGA 6.0 software.

RESULTS AND DISCUSSION

The whole genome sequence of *A. bisporus* was downloaded from NCBI database and scanned *in silico* for the presence of WRKY transcription factor and its variants. The results showed the presence of a number of WRKY domains distributed throughout the genome of *A. bisporus*. The priming sites were determined *in silico* using Genious 6.0 and tested various available WRKY primer pairs for the successful annealing. Out of ten primer pair tested three showed positive priming during the *in silico* analysis. These primers were tested under wet lab conditions against a number of *A. bisporus* strains and their fertile and non-fertile single spore isolates.

The WRKY primers pair A7G1F & A7G1R and NBS primer pair M13R1 & M1495R were tested in six strains and their 3 fertile and 3 non-fertile single spore isolates. Both the primer pairs successfully amplified WRKY transcription factor domains (Fig. 1 and Fig. 2). It was observed that the primers amplified the domain at different locations of the genome. Furthermore, the amplicon size also varied between 500 to 2000 bp. The results clearly demonstrated the presence of the factors at more than one location and also the structure and composition of the domain varied from one another.

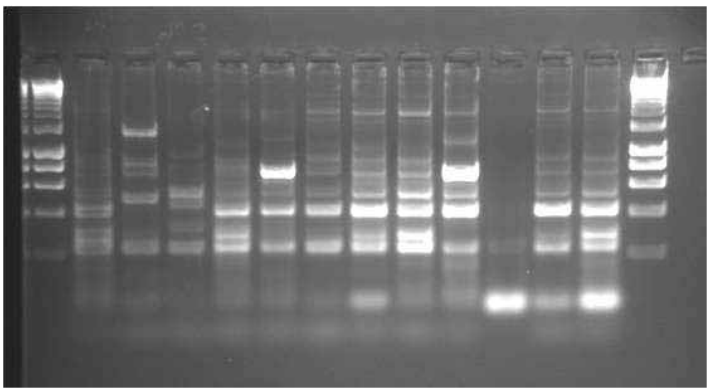

Figure 1. Amplification of WRKY transcription factor by primer pair A7G1F and A7G1R in different strains of *A. bisporus*

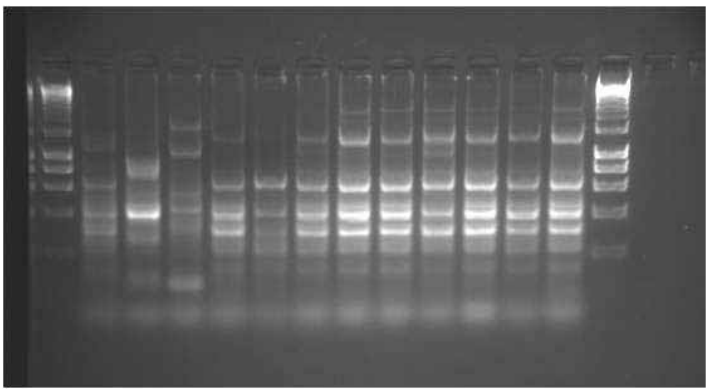

Figure 2. Amplification of WRKY transcription factor by primer pair M13R1 and M1495R in different strains of *A. bisporus*

The fragment were cut from the gel and eluted. The samples were sequenced using the WRKY primers and were subjected to BLAST against *A. bisporus* genome sequence from NCBI database (Fig 3). A total of 58 WRKY and related domains could be found distributed throughout the genome of *A. bisporus* (Fig 4).

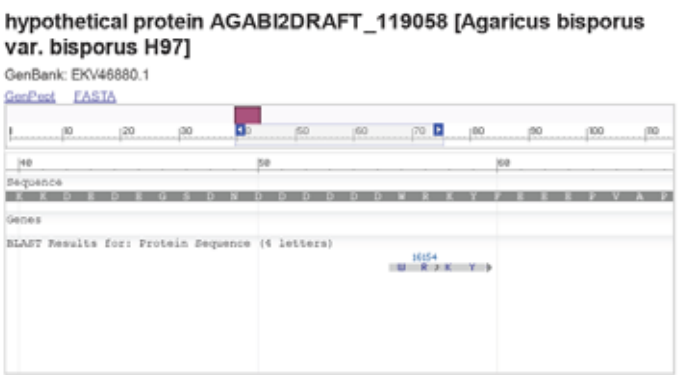

Figure 3. BLAST result of one of the WRKY transcription factor in *A. bisporus*

Earlier studies also showed the presence of different WRKY domains in plants and a few non-plant species. Different types of WRKY domains found in different plants and non-plant species are WRKYGQK, WRKYGEK, WRKYGKK, WKKYGQK, WQKYGQK, WSKYGQK, WSKYGQM, WTKYGQK, WNKYGQK, WKRKGQK, WVKYGQK, WRRYGLK, WRKYEDK, WRKYGKR, WRKYGSK, WEKFGEK, WRKYGQE, WKKYGHK, WRKCGLK, WRKYGQN, WKKYGYK, WKKYGED, WLKYGQK, WKKYEEK, WKKYGEK, WRKYGRK, WKKYGNK, WRKYGQT, WKKYGPK, WHKYGAK, WRKYGHK, WRKYGNK, WKNNGNT, WTKYDQR, WREYDQR, etc. (http://supfam.cs.bris.ac.uk/SUPERFAMILY/).

The presence of a number of WRKY like domains in the genome of *A. bisporus* indicates that different functions are to be performed by these genes. One of the Nucleotide binding site primers used in this study is of disease resistance gene. Presence of the domain also indicates the resistance to the biotic stress in mushrooms.

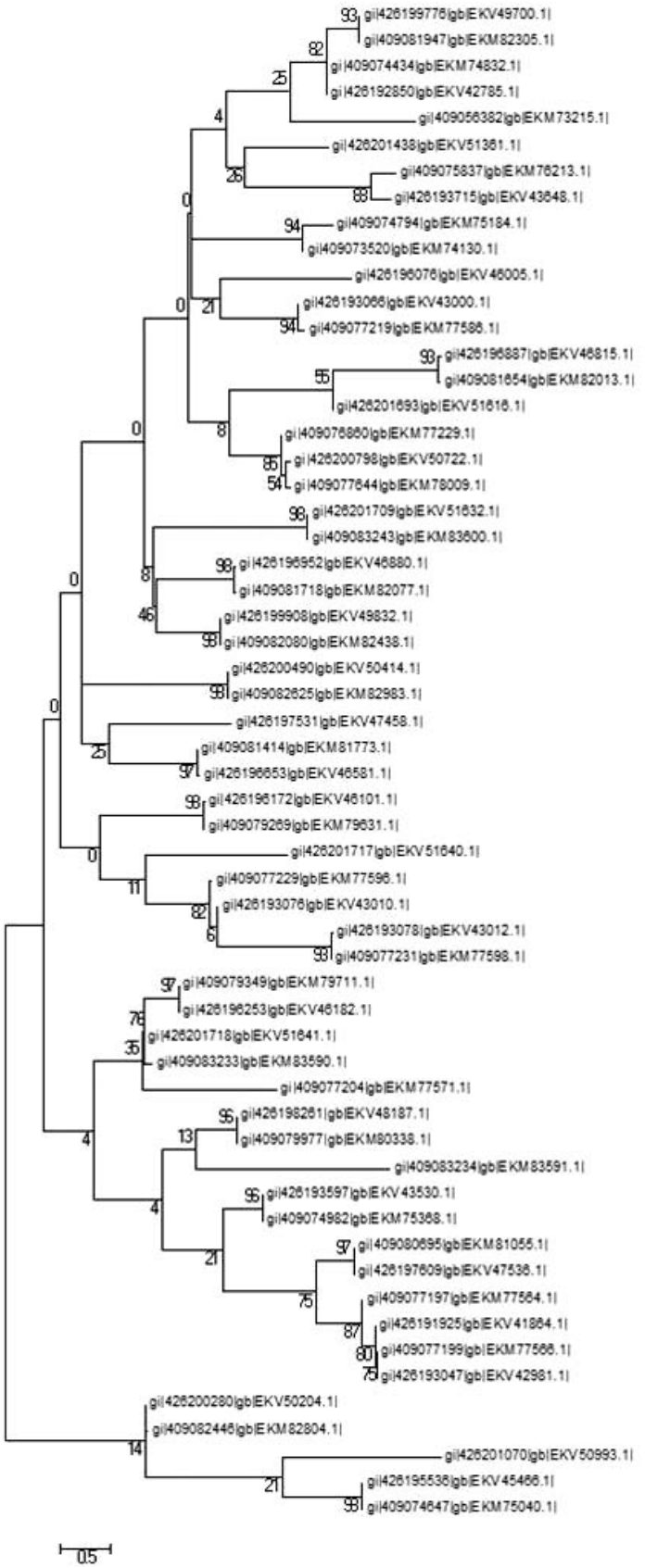

Figure 4. Phyologeny of WRKY Transcription factors distributed throughout the genome of *A. bisporus* using maximum likelyhood analysis

Transcriptional control is a major mechanism whereby a cell or organism regulates its gene expression. Sequence specific DNA-binding transcription regulators, one class of transcription factors, play an essential role in modulating the rate of transcription of specific target genes. In this way, they direct the temporal and spatial expressions necessary for normal development and proper response to physiological or environmental stimuli. Comparative genome analysis reveals that genes for transcription regulators are abundantly present in plant and animal genomes, and the evolution and diversity of eukaryotes seem to be related to the expansion of lineage-specific transcription regulator families. WRKY proteins are recently identified transcriptional regulators comprising a large gene family. The first cDNA encoding a WRKY protein, SPF1, was cloned from sweet potato (Ipomoea batatas). Numerous genes for WRKY proteins have since been experimentally identified from more than 10 other plant species, including *Arabidopsis thaliana* [5, 12], wild oats (*Avena fatua*) [15], orchardgrass (*Dactylis glomerata*) [16], barley (*Hordeum vulgare*) [17], tobacco (*Nicotiana tabacum*) [18-20], chamomile (*Matricaria chamomilla*) [21], rice (*Oryza sativa*) [17, 22], parsley (*Petroselinum crispum*) [7, 23], a desert legume (*Retama raetam*) [24], sugarcane (*Saccharum* hybrid cultivar) [25], bittersweet nightshade (*Solanum dulcamara*) [26], potato (*Solanum tuberosum*) [27, 28], and wheat (*Triticum aestivum*) [17]. In addition, over 70 WRKY genes were identified in the *Arabidopsis* genome by sequence similarity comparisons [29].

To date, WRKY genes have not been cloned from species other than plants. This is the first report on the presence of WRKY domains in higher basidiomycetes mushrooms. The characterization of the WRKY domains in mushroom is under progress.

REFERENCES

[1] Pandey SP and Somssich IE. (2009). The role of WRKY transcription factors in plant immunity. *Plant Physio*l. 150: 1648–1655.

[2] Eulgem T *et al*. (2000). The WRKY superfamily of plant transcription factors. *Trends Plant Sci*. 5: 199–206.

[3] Xie Z *et al*. (2005). Annotations and functional analyses of the rice WRKY gene superfamily reveal positive and negative regulators of abscisic acid signaling in aleurone cells. *Plant Physiol*. 137: 176–189.

[4] Eulgem T *et a*l. (1999). Early nuclear events in plant defence signalling: rapid gene activation by WRKY transcription factors. *EMBO J*. 18: 4689–4699.

[5] de Pater S *et al*. (1996). Characterization of a zinc-dependent transcriptional activator from *Arabidopsis*. *Nucleic Acids Res*. 24:4624-4631.

[6] Ishiguro S and Nakamura K. (1994). Characterization of a cDNA encoding a novel DNA-binding protein, SPF1, that recognizes SP8 sequences in the 50 upstream regions of genes coding for sporamin and beta-amylase from sweet potato. *Mol. Gen. Genet*. 244: 563–571.

[7] Rushton PJ *et al*. (1996). Interaction of elicitor-induced DNA-binding proteins with elicitor response elements in the promoters of parsley PR1 genes. *EMBO J*. 15: 5690–5700.

[8] Goff SA *et al*. (2002). A draft sequence of the rice genome *(Oryza sativa* L. ssp *japonica*). *Science* 296:92-100.

[9] Kato N *et al*. (2007). Identification of a WRKY protein as a transcriptional regulator of benzylisoquinoline alkaloid biosynthesis in *Coptis japonica*. *Plant Cell Physiol*. 48: 8–18.

[10] Zhang Y and Wang L. (2005). The WRKY transcription factor superfamily: its origin in eukaryotes and expansion in plants. *BMC Evol. Biol*. 5: 1

[11] Glockner G *et al*. (2002). Sequence and analysis of chromosome 2 of *Dictyostelium discoideum*. *Nature* 418:79 -785.

[12] Deslandes L *et al*. (2002). Resistance to *Ralstonia solanacearum* in *Arabidopsis thaliana* is conferred by the recessive *RRS1-R* gene, a member of a novel family of resistance genes. *PNAS* 99:2404-2409.

[13] Noutoshi Y *et al*. (2005). A single amino acid insertion in the WRKY domain of the *Arabidopsis* TIR-NBS-LRR-WRKY-type disease resistance protein SLH1 (sensitive to low humidity 1) causes activation of defense responses and hypersensitive cell death. Plant J. 43, 873–888

[14] Rushton Paul J *et al*. (2010). WRKY transcription factors. *Trends Plant Sci.* 15(5):247-258. doi: 10.1016/j.tplants.2010.02.006.

[15] Rushton PJ *et al*. (1995). Members of a new family of DNA-binding proteins bind to a conserved cis-element in the promoters of alpha-Amy2 genes. *Plant Mol Biol* . 29:691-702.

[16] Alexandrova KS and Conger BV. (2002). Isolation of two somatic embryogenesis- related genes from orchard grass (*Dactylis glomerata*). *Plant Sci*. 162:301-307.

[17] Sun C *et al*. (2003). A novel WRKY transcription factor, SUSIBA2, participates in sugar signaling in barley by binding to the sugar-responsive elements of the iso1 promoter. *Plant Cell* 15:2076-2092.

[18] Chen CH and Chen ZX (2000). Isolation and characterization of two pathogen- and salicylic acid-induced genes encoding WRKY DNA-binding proteins from tobacco. *Plant Mol Biol*. 42:387-396.

[19] Hara K *et al*. (2000) Rapid systemic accumulation of transcripts encoding a tobacco WRKY transcription factor upon wounding. *Mol Gen Genet*. 263:30-37.

[20] Wang ZP *et al*. (1998). An oligo selection procedure for identification of sequence- specific DNA-binding activities associated with the plant defence response. *Plant J*. 16:515-522.

[21] Ashida Y *et al*. (2002). Molecular cloning and mRNA expression of geraniol-inducible genes in cultured shoot primordia of *Matricaria chamomilla*. *Biosci Biotechnol Biochem*. 66:2511-2514.

[22] Kim CY *et al*. (2000).Identification of rice blast fungal elic- itor-responsive genes by differential display analysis. *Mol Plant Microbe Interact*. 13:470-474.

[23] Cormack RS *et al*. (2002). Leucine zipper-containing WRKY proteins widen the spectrum of immediate early elicitor-induced WRKY transcription factors in parsley. *Biochim Biophys Acta*. 1576:92-100.

[24] Pnueli L *et al*. (2002).: Molecular and biochemical mechanisms associated with dormancy and drought tolerance in the desert legume *Retama raetam*. *Plant J*. 31:319-330.

[25] Lambais MR. (2001). In silico differential display of defense-related expressed sequence tags from sugarcane tissues infected with diazotrophic endophytes. *Genet Mol Biol*. 24:103-111.

[26] Huang T and Duman JG. (2002). Cloning and characterization of a thermal hysteresis (antifreeze) protein with DNA-binding activity from winter bittersweet nightshade, *Solanum dulcamara*. *Plant Mol Biol*. 48:339-350.

[27] Beyer *et al*. (2001).Identification of potato genes induced during colonization by Phytophthora *infestans*. *Mol Plant Pathol*. 2:125-134.

[28] Dellagi A *et al*. (2000).A potato gene encoding a WRKY-like transcription factor is induced in interactions with *Erwinia carotovora* subsp *atroseptica* and *Phytophthora infestans* and is coregulated with class I endochitinase expression. *Mol Plant Microbe Interact*. 13:1092-1101.

[29] Dong J *et al*. (2003). Expression profiles of the *Arabidopsis* WRKY gene superfamily during plant defense response. *Plant Mol Biol*. 51:21-37.