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Identification and expression analysis of a new glycoside hydrolase family 55 exo-β-1,3-glucanase-encoding gene in *Volvariella volvacea* suggests a role in fruiting body development

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ABSTRACT

The edible straw mushroom *Volvariella volvacea* is an important crop in South East Asia and is predominantly harvested in the egg stage. Rapid stipe elongation and cap expansion result in a swift transition from the egg to elongation and maturation stage, which are subjected to fast senescence and deterioration. In other mushrooms, β-1,3-glucanases have been associated with degradation (softening) of the cell wall during stipe elongation and senescence. We present a new glycoside hydrolase family 55 (GH55) exo-β-1,3-glucanase gene, *exg2*, and highly conserved deduced EXG2 protein. The 3D model and presumed catalytic residues of *V. volvacea* EXG2 are identical to *Lentinula edodes* EXG2 and *Phanerochaete chrysosporium* Lam55A, supporting similar enzymatic functions. In addition to previous association to stipe elongation and senescence, our data clearly indicates a role for cap (pileus) expansion. Digital gene expression, quantitative PCR and isobaric tags for relative and absolute quantification analysis showed low *exg2* and EXG2 levels in primordia, button, egg and elongation stages and significantly increased levels in the maturation stage. Subsequent relative quantitative PCR analysis designated expression of *exg2* to the stipe in the elongation stage and to the pileus and stipe in the maturation stage. EXG2 cell wall softening activity, close correlation of *exg2* expression with the principal expanding mushroom tissues and a strong conservation of expression patterns and protein sequences in other mushrooms, make *V. volvacea exg2* an important candidate for future studies on mechanisms of fruiting body expansion and senescence causing commodity value loss.

1. Introduction

Volvariella volvacea (Bull, Ex. Fr.) Sing., also known as the Chinese straw mushroom, is cultivated on a large scale in many tropical and subtropical regions of Southeast Asia because of its popularity in the diets of these regions (Ding et al., 2007). *V. volvacea* is harvested in its egg stage which is characterized by a pileus and stipe that are still fully enclosed by the universal veil, giving the mushroom its egg shape appearance (Chang and Yau, 1971). In the next stage, the elongation stage, the stipe rapidly extends to near full length in a short period of several hours, whereas the pileus remains folded. This is coupled to rupturing of the exo-pellicle (thus forming the volva) and closely followed by expansion of the pileus and sporulation in the maturation stage. Once entering the elongation and maturation stage, *V. volvacea* experiences a fast rate of senescence and deterioration, and has a considerably shorter shelf life than in the egg stage (Mau et al., 1997). The transition period from egg stage to elongation and maturation stage comprises a mere 3 to 24 h (variation occurs between different strains) which makes it important to identify the genes involved in this rapid morphogenesis for improvement of future production of *V. volvacea*.

Fungal cell walls are composed of chitin, β-glucans and several other polymers (Bowman and Free, 2006) and experience major structural changes and remodeling during morphogenesis (Adams, 2004). β-Glucans, that contribute for an important part to the rigidity and strength of the cell wall (Latgé, 2007), were found in very high proportions in polysaccharide extracts of *V. volvacea* fruiting bodies (Yang et al., 2003). Exo-β-1,3-glucanases that typically hydrolyze β-glucan chains by cleaving glucose residues from the non-reducing end (Martin et al., 2007) and, by degrading β-glucan in the cell wall, have been suggested

Abbreviations: GH55, glycoside hydrolase family 55; CTAB, Hexadecyl trimethyl ammonium Bromide; PR, primordia; BU, button stage; EG, egg stage; EL, elongation stage; MA, maturation stage; DGE, digital gene expression; Q-PCR, real time quantitative PCR; iTRAQ, isobaric tags for relative and absolute quantification; ORF, open reading frame; cDNA, DNA complementary to RNA; AA, amino acid; CDS, sequence coding for amino acids in protein; TPM, transcripts per million clean tags.

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to take part in the morphogenesis of fungi (Adams, 2004; Stubbs et al., 1999). Moreover, studies on *exg2* of the mushroom *Lentinula edodes*, belonging to the same order (*Agaricales*) as *V. volvacea*, indicated that glycoside hydrolase family 55 (GH55) exo-β-1,3-glucanase has a dual function, in stipe elongation and in fruiting body senescence (Sakamoto et al., 2005, 2012). *L. edodes exg2* was found to be highly expressed especially in the stipe, yet only weakly in gills and the pilei of the mature fruiting bodies. Because rupturing of the volva and hence reduction in crop value of *V. volvacea* are initially caused by stipe elongation, we focused on the presence and expression of GH55 exo-β-1,3-glucanases in stipes of developing *V. volvacea* fruiting bodies.

Except for *L. edodes exg2* (Sakamoto et al., 2005) and *Phanerochaete chrysosporium Lam55A* (Kawai et al., 2006), little is known about GH55 exo-β-1,3-glucanases in *Basidiomycetes*. GH55 exo-β-1,3-glucanase genes remain unidentified in most fungi and have not yet been isolated or analyzed. Here, we describe for the first time, the cloning, identification and expression analysis of *V. volvacea* GH55 exo-β-1,3-glucanase-encoding gene *exg2*.

2. Materials and methods

2.1. Organisms and growth condition

V. volvacea H1521 was obtained from the Agricultural Culture Collection of China (ACCC52633), and maintained with periodic transfers on potato dextrose agar, at 20 °C. For liquid cultures, H1521 was cultivated in potato dextrose medium at 33 °C while shaking at 120 rpm. For solid cultures, producing fruiting bodies, H1521 was cultivated on rice straw compost according to Chen's method (Chen et al., 2004). Samples for this study were taken at different stages of mushroom development: primordia (PR, day 8 after inoculation), button stage (BU, day 10 after inoculation), egg stage (EG, day 13 after inoculation), elongation stage (EL, day 13.5 after inoculation) and maturation stage (MA, day 14 after inoculation). For the primordia, whole young fruiting bodies (pileus and stipe cannot yet be distinguished) were sampled. From the button, egg, elongation and maturation stages stipes (pileus and stipe can be distinguished) were sampled. For the subsequent tissue specific expression analysis of *exg2*, new stipe, pileus and volva of the elongation and maturation stage were sampled. All samples were immediately frozen in liquid nitrogen before DNA/RNA extraction.

2.2. DNA and RNA sample preparation

DNA samples for gene sequence confirmation were obtained from the mycelium of H1521 using an improved Hexadecyl trimethyl ammonium Bromide (CTAB) method (Porebski et al., 1997). RNA samples from different fruiting body stages were prepared using the RNeasy plant mini kit (QIAGEN, Germany) according to the manufacturer's protocol. cDNA used for gene model confirmation was synthesized from total RNA, using the RNA LA PCR Kit Ver.1.1 (TAKARA, Japan) according to the manufacturer's protocol.

2.3. Identification of V. volvacea exg2

Based on *V. volvacea* whole genome sequencing data of strain PYd21 (ACCC52632), we performed gene prediction and found a putative *V. volvacea* GH55 exo-β-1,3-glucanase-encoding gene (*exg2*) through local BLAST search with *L. edodes exg2*. In order to fill gaps that had remained in the database sequence, six pairs of primers (Supplemental Table S1) were designed for subcloning using segmented-PCR (the 5605 bp-gDNA sequence was segmented into 6 fragments: G-1, G-2, G-3, G-4, G-5 and G-6, containing approximately 100 to 200 bp reciprocal overlap) and sequence confirmation.

2.4. Analysis of V. volvacea exg2 and EXG2

Transcriptome sequence raw reads were mapped against *V. volvacea exg2* using ZOOM software (Zhang et al., 2010) for identification of exons and introns. The predicted gene model was confirmed through sequencing of the complete cDNA obtained by PCR with primers *exg2*F and *exg2*R (Supplemental Table S1). We used ExPASy Protparam (http://www.expasy.ch/tools/protparam.html) to analyze basic physical and chemical properties of *V. volvacea* EXG2; SignalP 4.0 (http://www.cbs.dtu.dk/services/) to predict signal peptides; TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) to predict transmembrane regions and orientation; PSORT (http://psort.hgc.jp/) to analyze subcellular localization and InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/) to analyze conserved domains. We compared *V. volvacea* EXG2 with *L. edodes* EXG2 and *P. chrysosporium* Lam55A using the secondary and three dimensional structure prediction program Phyre 2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) (Kelley and Sternberg, 2009) and Swissmodel (http://swissmodel.expasy.org/) (Arnold et al., 2006), respectively. Multiple alignments were done using ClustalX 1.83 and assembly of the phylogenetic tree of GH55 exo-β-1,3-glucanases was done using MEGA 5.0 software. Evolutionary relationships were inferred by the neighbor-joining method and a bootstrap of 1000 replications. Evolutionary distances were computed using the Poisson correction method.

2.5. Transcription pattern analysis of V. volvacea exg2

Digital gene expression (DGE) was used for transcription pattern analysis of *V. volvacea exg2*. Briefly, the transcription level of *exg2* is defined as the normalized number of unambiguous tags referred to as number of transcripts per million clean tags (TPM). TPM values are computed by dividing the number of clean tags mapped on the target gene by the total number of clean tags measured in each sample, multiplied by 1,000,000 (Morrissy et al., 2009; 't Hoen et al., 2008). Final *exg2* relative transcription levels were calculated using denary logarithm values of TPM. Real time quantitative PCR (Q-PCR) was used to verify DGE results. RNA for cDNA cloning was also used for Q-PCR using the primers *exg2*QF and *exg2*QR (Supplemental Table S1). The glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was used as an internal control gene (primers listed in Supplemental Table S1). Q-PCR was done using a CFX96 Real-Time PCR Detection System (BIO-RAD, USA) with SsoAdvanced SYBY Green Supermix (BIO-RAD, USA). The $2^{-\Delta\Delta Ct}$ method was used for Q-PCR data analysis (Livak and Schmittgen, 2001).

2.6. Expression pattern analysis of V. volvacea EXG2

The expression pattern of *V. volvacea* EXG2 was analyzed based on proteome expression analysis using isobaric tags for relative and absolute quantification (iTRAQ, Wiese et al., 2006). Five samples, five developmental stages (primordia, button, egg, elongation and maturation), had been labeled 116, 117, 118, 121 and 119 for proteome analysis respectively, and were analyzed with a Q-Exactive mass spectrometer. Data was interpreted using PEAKS 6 software (Ma et al., 2003). The relative amounts of EXG2 in various samples were determined using weighted averages of the amounts of several peptides mapped on the reference sequence from PEAKS, using default parameters.

2.7. Sequence accessions

The draft genome of *V. volvacea* PYd21 (ACCC52632) is available at NCBI: PRJNA171553. Digital gene expression data can be found at NCBI: GSM1060232-1060236, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=pxcbpqmmwqeuopo&acc=GSE43297. The 5.6 Kb genome fragment, CDS and deduced EXG2 protein of gene *exg2* are available at Genbank: JX412948. The 2511 cDNA sequence was deposited under GenBank: JX412949 and the EXG2 protein under GenBank: AFS68743.