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ORIGINAL ARTICLE

Gene expression metadata analysis reveals molecular mechanisms employed by *Phanerochaete chrysosporium* during lignin degradation and detoxification of plant extractives

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Abstract Lignin, most complex and abundant biopolymer on the earth's surface, attains its stability from intricate polyphenolic units and non-phenolic bonds, making it difficult to depolymerize or separate from other units of biomass. Eccentric lignin degrading ability and availability of annotated genome make *Phanerochaete chrysosporium* ideal for studying lignin degrading mechanisms. Decoding and understanding the molecular mechanisms underlying the process of lignin degradation will significantly aid the progressing biofuel industries and lead to the production of commercially vital platform chemicals. In this study, we have performed a large-scale metadata analysis to understand the common gene expression patterns of *P. chrysosporium* during lignin degradation. Gene expression datasets were retrieved from NCBI GEO database and analyzed using GEO2R and Bioconductor packages. Commonly expressed statistically significant genes among different datasets were further considered to understand their involvement in lignin degradation and detoxification mechanisms. We have observed three sets of enzymes commonly expressed during ligninolytic conditions which were later classified into primary ligninolytic, aromatic compound-degrading and other necessary enzymes. Similarly, we have observed three sets of genes coding for detoxification and stress-responsive, phase I and phase II metabolic enzymes. Results obtained in this study indicate the coordinated action of enzymes involved in lignin depolymerization and detoxification-stress responses under ligninolytic conditions. We have developed tentative network of genes and enzymes involved in lignin degradation and detoxification mechanisms by *P. chrysosporium* based on the literature and results obtained in this study. However, ambiguity raised due to higher expression of several uncharacterized proteins necessitates for further proteomic studies in *P. chrysosporium*.

Keywords *Phanerochaete chrysosporium* · Transcriptome · Lignocellulose · Gene expression omnibus (GEO) · GEO2R · Detoxification responses · Phase I and phase II metabolic enzymes

Introduction

Naturally, wood is composed of two organic compound groups (a) carbohydrates (65–75%) and (b) lignin (20–30%) along with organic extraneous compounds (4–10%) and inorganic minerals (calcium, potassium, etc.) (Nascimento et al. 2013). Lignin constitutes second most abundant biopolymer, found in closer associations with cellulose and hemicellulosic units, complex polyphenolic units and non-phenolic linkages provide lignin with high stability. Lignin is considered as major bottleneck in biofuel industries, as it is necessary to separate lignin from other units of biomass for the efficient production of biofuels. Also, if degraded efficiently lignin can be used to produce commercially important platform chemicals. Only few microorganisms were reported till today with efficient lignin degrading abilities. *Phanerochaete chrysosporium* is a wood-degrading

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white rot fungus belonging to Basidiomycetes fungi, well known for its eccentric lignin degrading ability (Randall and Reddy 1992; Singh and Chen 2008). Apart from lignin, *P. chrysosporium* also exhibits great ability in degrading and mineralizing various synthetic dyes, organic pollutants such as 2,4-dichlorophenol, 2,4-dinitrotoluene, endosulfan, pentachlorophenol, phenanthrene and several other harmful organic chemicals (Kullman and Matsumura 1996; Reddy and Gold 2000; Valli et al. 1992; Valli and Gold 1991). Degrading ability of *P. chrysosporium* is credited to its highly efficient enzyme system; it secretes a wide range of oxidative and hydrolytic enzymes for the successful degradation of various organic compounds. Several studies have reported about the lignin-degrading enzyme system of *P. chrysosporium*, which majorly consists of ligninolytic peroxidases such as lignin peroxidase (LiP), manganese peroxidase (MnP) and hydrogen peroxide generating enzymes. *P. chrysosporium* induces its enzyme arsenal under nutrient-deficient culture conditions during its secondary metabolism (Keyser et al. 1978). Studies have reported that *P. chrysosporium* enzyme system can catalyze the primary oxidation of various persistent xenobiotic compounds such as chloroaromatic, polyaromatic compounds and dioxins (Hammel et al. 1986).

Martinez et al. (2004) has sequenced and annotated the whole genome of *P. chrysosporium*, in fact it was the first whole genome sequence from phylum Basidiomycota (Martinez et al. 2004). The 30 Mb haploid genome of *P. chrysosporium* with 11,777 protein coding genes has revealed several significant facts about the genes and enzymes involved in wood decaying process (Martinez et al. 2004). Genome of *P. chrysosporium* majorly codes for cytochrome P450 superfamily, glucose methanol choline oxidoreductases, protein kinases, alcohol oxidases, short-chain reductases, aspartyl proteases, von Willebrand factor, lectin type proteins and several other ligninolytic enzymes (Figure S1). Present-day annotated genome of *P. chrysosporium* RP-78 v2.2 encodes about 444 CAZymes [glycoside hydrolase (181), glycosyl transferases (70), auxiliary activity (89), carbohydrate binding domains (65), carbohydrate esterases (20) and polysaccharide lyases (6)] out of which auxiliary activity, carbohydrate esterases and glycosyl transferases were found during lignin degradation. (Martinez et al. 2004; Wymelenberg et al. 2006) (Figure S2). Genome level transcriptome studies conducted during last decade has revealed several significant facts about the differential expression patterns of several genes involved in lignin depolymerization.

Minami et al. (2007) have performed a LongSAGE analysis on *P. chrysosporium* to understand the changes in transcriptome during the initiation of manganese peroxidase and lignin peroxidase enzymes. LongSAGE analysis has revealed about several candidate gene sequences involved in regulation of the LiP and MnP enzyme production (Minami et al. 2007) (GSE6649). Wymelenberg et al. (2009) have studied the extracellular protein products of *P. chrysosporium* grown under nutrient-limited and replete conditions. Results obtained by them have confirmed the expression of several lignocellulose degrading enzymes under nutrient-limited conditions and also reinforced the role of novel proteins (Wymelenberg et al. 2009) (GSE14735). To study the extracellular proteins secreted by *P. chrysosporium* under standard cellulolytic conditions, Wymelenberg et al. (2009) have performed a whole transcriptome study. This gene expression study has confirmed the significance of carbohydrate active enzymes and supported the function of many novel proteins involved in lignocellulose degradation (Wymelenberg et al. 2009, 2010) (GSE14734). Wymelenberg et al. (2011) have conducted transcriptome study to analyze the gene expression patterns of *Postia placenta* and *Phanerochaete chrysosporium* colonized on (aspen) *Populus grandidentata* and (pine) *Pinus strobus*. Results have showed that transcriptome of these fungi is significantly influenced by wood species and this study has majorly differentiated molecular mechanisms employed by white and brown rot decay patterns (Wymelenberg et al. 2011) (GSE27941). Thuillier et al. (2014) have performed a transcriptome study of *Phanerochaete chrysosporium* cultured on oak acetonic extracts and reported that *P. chrysosporium* employs both intracellular antioxidative detoxification mechanisms along with extracellular enzymes for lignin degradation. This study has also revealed the functional characteristics of PcGTT2.1, a glutathione-*S*-transferase isoform, involved in reducing the cellular toxicity caused by lipid peroxidation and also reported the loss of GTT2.1 isoform in some of the non-wood-decaying fungi (Thuillier et al. 2014) (GSE54542). Gaskell et al. (2014) have conducted experiments to understand the gene expression patterns of *P. chrysosporium*, colonized on hybrid poplar (*Populus alba x tremula)*, 82 and 64 transgenic derivatives (syringyl rich). The microarray results have showed that gene expression patterns of *P. chrysosporium* are considerably influenced by lignin composition of the growth substrate, especially peptides corresponding to various oxidoreductases were found to be highly expressed in 82 and 64 transgenic line substrates (Gaskell et al. 2014) (GSE52922). Korripally et al. (2015) have performed a whole transcriptome study of *P. chrysosporium* by culturing it on spruce wood samples with an efficient oxidant sensing beads at 40 and 96-h incubation periods. This study has also revealed the functional properties of 72 unknown proteins available under the *P. chrysosporium* genome database v2.2, cytochrome P450 monooxygenases and transporters (Korripally et al. 2015) (GSE69461). Skyba et al. (2016) have performed a whole transcriptome study of *P. chrysosporium* and *P. placenta*,

cultured on three *Populus trichocarpa* (poplar) wood substrates with different chemical compositions (Skyba et al. 2016). This study has clearly showed the influence of growth substrate (wood composition) and incubation period on the gene expressions of *P. chrysosporium* and *P. placenta* (Skyba et al. 2016) (GSE69008). All the above genome-wide studies have significantly influenced the present-day understanding about the plant cell wall degrading abilities of *P. chrysosporium* and revealed functional properties of several uncharacterized proteins.

During the process of wood degradation, *P. chrysosporium* is exposed to various highly toxic phenolic plant extractives such as flavonoids, quinones, stilbenes and tannins (Table-SI) (Nascimento et al. 2013; Shalaby and Horwitz 2015; Thuillier et al. 2014). Among these plant extractives, flavonoids and stilbenes possess strong antifungal properties and are also required for the durability of wood (Feraydoni and Hosseinihashemi 2012; Harborne and Williams 2000; Nascimento et al. 2013). However, the ligninolytic white rot fungi have developed an efficient enzyme system involved in antioxidant and detoxification mechanisms (Thuillier et al. 2014). Majorly the detoxification system of white rot fungi can be classified into phase I (cytochrome P450 group, epoxide hydrolases) and phase II (glutathione-*S*-transferase, quinone oxidoreductase, UDP-glucuronosyltransferases) enzyme systems. These enzyme systems are specifically induced by a variety of xenobiotics and plant extractive compounds (Doddapaneni and Yadav 2005). Thus, most of the white rot fungi maintain a complementary system of extracellular enzymes involved in wood degradation and a simultaneous intracellular antioxidant and detoxification systems.

Studies have reported that *P. chrysosporium* is equipped with a large number of genes coding for cytochrome P450 monooxygenases, phase I and phase II metabolic enzymes and signaling cascade genes (Doddapaneni and Yadav 2005). In the last decade, extensive research has been performed on the cytochrome P450 monooxygenases and its role in several physiological and catalytic processes of *P. chrysosporium* such as ligninolysis, secondary metabolism and xenobiotic degradation processes. Doddapaneni and Yadav (2005) has performed global gene expression studies of *P. chrysosporium* to explain the differential expression patterns of cytochrome P450 monooxygenases using a custom-designed 70-mer oligonucleotide microarray. For the first time this study has proved the involvement of cAMP and MAP kinase signaling pathways during the biodegradation and secondary metabolism of *P. chrysosporium* (Doddapaneni and Yadav 2005). Later, studies have also reported that cAMP and calmodulin (CaM) signaling mechanisms play crucial role in expression of ligninolytic peroxidases, as expression of calmodulin inhibitor W-7 in *P. chrysosporium* has resulted in regulation of *lip* and *mnp* genes and their isoforms (Sakamoto et al. 2010; Suetomi et al. 2015). Subramanian and Yadav (2009) have proved the significance of P450 monooxygenases in degradation of nonylphenol (endocrine-disrupting chemical) under different nutrient conditions using a custom-designed microarray. This study has shown the involvement of P450 monooxygenases in nonylphenol degradation (Subramanian and Yadav 2009). In the year 2009, Jiang et al. has performed genome-wide expression analysis specifically for identifying the genes involved in secondary metabolism of *P. chrysosporium*. This study has revealed the expression of genes coding for enzymes such as aryl alcohol dehydrogenase, cytochrome P450, alkyl hydroperoxide reductase, catalase, and ABC transporters (Jiang et al. 2009). Subramanian and Yadav (2009) have reported the transcriptome profiles of cytochrome P450 in *P. chrysosporium* under varied nutrient conditions (Subramanian and Yadav 2008). Expression of P450 enzymes under different nutrient conditions suggests the role of P450 enzymes in the catalytic activity of *P. chrysosporium*. Differential expression of certain P450 enzymes during low- and high-nutrient conditions reveals the specific role played by these enzymes under ligninolytic and non-ligninolytic conditions (Subramanian and Yadav 2008). Chigu et al. (2010) have performed transcriptomic profiling of *P. chrysosporium* cytochrome P450 monooxygenases (PcCYPS) involved in anthracene metabolism (Chigu et al. 2010). This study has revealed that 14 PcCYP genes are involved in step by step conversion of anthracene to anthraquinone. 12 PcCYPS are up-regulated upon exogenous addition of anthracene. Out of 12 PcCYPS, 5 genes showed high catalytic activity against anthracene and also reported that these genes play major role in in vivo anthracene metabolism (Chigu et al. 2010).

In this study, we have conducted a large-scale metadata analysis on *P. chrysosporium* gene expression datasets, specifically to demonstrate the common gene expression patterns involved during extracellular lignin degradation and intracellular detoxification mechanisms. To the extent of our knowledge this is the first report on metadata analysis of *P. chrysosporium* for demonstrating lignin degradation and detoxification and stress-responsive mechanisms.

Data retrieval and methodology

Data retrieval

We have used the term *P. chrysosporium* to search for the gene expression datasets available in NCBI Gene expression omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/), a public repository for gene expression datasets. All the gene expression dataset corresponding to *P. chrysosporium* listed under "GEO Datasets" window was carefully

analyzed by accessing experimental information provided by corresponding research team under "Accession display" window. Totally, there are ten gene expression datasets which are currently available in GEO database out of which we have retrieved total of 8 *P. chrysosporium* gene expression datasets (six were microarray datasets, one RNA sequencing and one LongSAGE dataset). The NCBI GEO accession IDs of gene expression datasets retrieved were GSE14734, GSE14735, GSE54542, GSE27941, GSE52922, GSE69008, GSE69461 and GSE6649. Substrate and platform level details of these gene expression datasets are shown in Table 1. We have specifically considered *P. chrysosporium* gene expression datasets which were based on the natural plant biomass and simple synthetic compounds containing growth mediums to monitor the change in gene expression patterns under ligninolytic conditions.

Data analysis

The microarray datasets retrieved were analyzed using GEO2R (an R-based interactive online tool) (https://www.ncbi.nlm.nih.gov/geo/info/geo2r.html) and Bioconductor packages GEOquery and limma based on R software version 3.2.2. The settings used in GEO2R for analyzing the microarray datasets were listed below autodetect option (for log transformation of the data), box–whisker plot (samples and value distributions) and submitter provided annotations (for gene level annotations). The experimental design and sample grouping information were obtained from the gene expression datasets and the corresponding literature. Top 250 function was used to obtain the differentially expressed genes statistically significant genes, the Top 250 function internally uses limma (linear models for microarray data) for the statistical analysis and the genes are ranked based on their *p* values. The differentially expressed genes were obtained after performing Benjamini and Hochberg false discovery rate multiple testing correction method with a *p* value 0.05. The profile graphs were obtained for the differentially expressed genes, which were reported in the supplementary file-S1 (Figure S3). As we have mentioned earlier, the process of biological contextualization was based on the supplier provided annotations and supplementary information which included mainly InterPro Hits and Protein IDs. The gene and protein level annotations of *P. chrysosporium* were also obtained from MycoCosm (fungal genome repository) (Grigoriev et al. 2011, 2013). We have also used other analysis options available in JGI-MycoCosm such as Gene Ontology (GO) (Botstein et al. 2000; Consortium 2015), EuKaryotic Orthologous Groups (KOG) (Tatusov et al. 2003) and CAZy (Cantarel et al. 2009; Lombard et al. 2014) for analyzing the results obtained. The differentially expressed genes were also represented as hierarchical clusters using the Cluster 3.0 software (de Hoon et al. 2004) based on cluster for both genes and arrays using the complete linkage options, thus obtained cluster output files were used for visualization and development of dendrograms using Java Treeview software using the standard conditions (Saldanha 2004) (Figure S4). We have used Venny 2.1 (Oliveros 2007) and Jvenn (Bardou et al. 2014) softwares to get the common differentially expressed gene lists among different datasets. We have retrieved sample

Table 1 Details of the *P. chrysosporium* transcriptome metadata retrieved from NCBI GEO and NCBI SRA

GEO-ID	Platform and technology	Substrate	#Samples	References
GSE54542	NimbleGen *Phanerochaete chrysosporium* arrays	Oak acetonic extractives	6	Thuillier et al. (2014)
GSE27941	NimbleGen *Phanerochaete chrysosporium* arrays	Ball-milled aspen, Ball-milled pine	6	Wymelenberg et al. (2011)
GSE52922	NimbleGen *Phanerochaete chrysosporium* arrays	P717 hybrid line, Transgenic line 82 Transgenic line 64	9	Gaskell et al. (2014)
GSE14734	NimbleGen *Phanerochaete chrysosporium* arrays	Cellulose, Glucose, Ball-milled aspen	9	Wymelenberg et al. (2009, 2010)
GSE14735	NimbleGen *Phanerochaete chrysosporium* arrays	Replete medium Carbon limited Nitrogen limited	9	Wymelenberg et al. (2009, 2010)
GSE69008	NimbleGen *Phanerochaete chrysosporium* arrays	Poplar wood substrates	24	Skyba et al. (2016)
GSE6649	Long Serial analysis of gene expression	Basal III medium (1% (v/v), 1% (w/v) glucose, 20 mM Na-2,2-dimethylsuccinate (pH 4.5), 0.0001% thiamine, and 1.2 mM ammonium tartrate) 3 mM veratryl alcohol	2	Minami et al. (2007)
GSE69461	Illumina HiSeq 2000	*Picea glauca* (spruce sapwood)	18	Korripally et al. (2015)

level RPKM (Reads Per Kilobase of transcript per Million mapped reads) values from the supplementary information of GSE69461 dataset (Korripally et al. 2015). Further statistical analysis was performed on the sample level RPKM values using edge R (McCarthy et al. 2012; Robinson et al. 2010; Robinson and Smyth 2008; Zhou et al. 2014), limma (Ritchie et al. 2015) and Glimma Bioconductor packages Glimma (http://bioconductor.org/packages/release/bioc/html/Glimma.html) to obtain statistically significant differentially expressed genes, reported in supplementary material-S2. From the obtained results genes encoding for ligninolytic and detoxification, stress-responsive pathways were specifically retrieved based on their InterPro annotations. Sample level data of GSE6649 (LongSAGE) dataset was retrieved from NCBI GEO database and was further analyzed using Identification of Differentially Expressed Genes 6 (IDEG6), orphan tags were removed (sequential errors) and further statistical analysis was performed using Audic-Claverie test, as reported by (Minami et al. 2009). We have reported the process of data analysis earlier in our previous work which reported the metabolic and molecular gene networks employed by *P. chrysosporium* during cellulose and hemicellulose degradation (Kameshwar and Qin 2017).

Summary of data analysis

Based on the growth substrates used for culturing of *P. chrysosporium*, the gene expression datasets considered for the present study were divided as customized synthetic growth medium (containing cellulose, glucose or other commercially available nutrients supplemented with Highley's basal medium) and complex natural plant biomass medium (containing ball-milled aspen, ball-milled pine, spruce wood and poplar wood substrates) reported earlier (Kameshwar and Qin 2017). The accession IDs of gene expression datasets belonging to customized synthetic growth mediums were GSE14734 [HBM supplemented with 0.5% (wt/vol) of BMA or cellulose or glucose as sole carbon source), GSE14735 (replete B3 medium, carbon-limited medium and nitrogen-limited medium] (Wymelenberg et al. 2009, 2010) and GSE6649 (Minami et al. 2007). The GEO accession IDs of complex natural plant biomass medium were GSE27941 (0.5% of ball-milled aspen and ball-milled pine as the sole carbon source supplemented with HBM) (Wymelenberg et al. 2011), GSE52922 [parental hybrid clone line *Populus trichocarpa* P717 (65 mol% of syringyl), transgenic line 64 (94% syringyl) and transgenic line 82 with (85 mol% of syringyl)] (Gaskell et al. 2014), GSE54542 (fine-powdered oak heartwood samples extracted using acetone and further resuspended in DMSO followed by a set of extraction processes) (Thuillier et al. 2014), GSE69461 (microtomed tangential sections of *Picea glauca* coated with 90 µl of agar supplemented with nitrogen mineral salt medium) (Korripally et al. 2015). Similarly GSE69008 contained chemically distinct *Populus trichocarpa* wood substrates: high lignin–low glucose (A), low lignin–high glucose (B) and average lignin–average glucose (C) (Skyba et al. 2016). The detailed explanation about the experimental conditions and growth protocols performed for the gene expression study protocol can be followed from the corresponding literature cited (Figure S6).

Results

Lignin-oxidizing and auxiliary enzymes

Degradation of lignin and its derivatives is the subject of interest since several years. Advancement of high-throughput genomic and proteomic methods in the recent years has revealed various significant facts about lignin degradation mechanisms employed by *P. chrysosporium*. According to Kirk et al. (1987), an array of oxidases and peroxidases is secreted by white rot fungi for the initial degradation of lignin, these reactions release highly reactive and non-specific free radicals which leads to a complex series of spontaneous cleavage reactions (Alic et al. 1991; Kirk and Farrell 1987; Martinez et al. 2004). Most of the Basidiomycetes fungi and especially white rot fungi secrete extracellular laccases, which are involved in the single electron oxidation of phenols, phenoxy radicals, aromatic amines and electron-rich compounds, ultimately transferring four electrons to O_2 and reducing it to H_2O molecule (Kirk and Farrell 1987; Thurston 1994). The whole genome sequencing studies have showed that *P. chrysosporium* does not code for any conventional laccase; however, it codes for a cluster of four multicopper oxidases (MCO) and ferroxidase enzymes. Thus, multicopper oxidases and ferroxidases secreted by *P. chrysosporium* are involved in extracellular oxidation of lignin along with other lignin-oxidizing enzymes (Martinez et al. 2004). *P. chrysosporium* genome consists of ten *lip* genes coding for lignin peroxidases, five *mnp* genes coding for manganese peroxidase and 1 hybrid peroxidase (pc.91.32.1) encoding gene sequences (Martinez et al. 2004). From the metadata analysis, we have observed that expression of peroxidase-encoding genes in *P. chrysosporium* varies differentially based on the source of nutrients and time of infection. Gene expression studies mainly GSE14735, GSE69008, GSE69461 and GSE52922 have provided a significant evidence on differential expression of the ligninolytic peroxidases. Earlier studies have reported the differential expression of lignin peroxidase under nutrient-limited conditions, but the mechanism

behind its expression is not clear. Current metadata analysis supports previous findings on the expression of lignin-degrading peroxidases in *P. chrysosporium* both in synthetic and natural supplemented medium.

Fungal lignin peroxidase-coding genes were highly expressed when *P. chrysosporium* was cultured on ball-milled aspen, nitrogen and carbon-limited medium, hybrid line P717, transgenic line 82, transgenic line 64, control, high lignin–low glucose, day 3 cultures of GSE6649 and spruce wood at 96 h of incubation. The genes encoding manganese peroxidases were only found to be highly expressed in spruce wood 96-h incubation sample. Animal haem peroxidases were expressed in cellulose, carbon- and nitrogen-limited, ball-milled aspen, transgenic line 82, hybrid line P717, and low lignin–high glucose, average lignin–average glucose mediums. Chloroperoxidases were highly expressed in ball-milled aspen, cellulose growth medium, transgenic line 64. GMC oxidoreductases coding genes were highly expressed in ball-milled aspen, cellulose medium, transgenic line 64, high lignin–low glucose and 40-h incubation period. Glyoxal coding genes were highly expressed in cellulose, glucose, carbon-limited nutrient mediums. Multicopper oxidase-encoding genes were differentially expressed in spruce wood incubated for 96 h under high lignin–low glucose conditions. Genes encoding amine oxidase or flavin amine oxidase, copper amine oxidase were differentially expressed in high lignin–low glucose, average lignin–average glucose, nitrogen-limited conditions, and 40-h incubation period growth samples. Genes encoding for copper radical oxidase and aryl alcohol dehydrogenase were found to be highly expressed in 40-h incubation period samples.

Delta-9 acyl-CoA desaturase which is involved in unsaturated fatty acid biosynthesis was found to be highly expressed in nitrogen-limited, glucose, cellulose, ball-milled pine, 64 and 82 transgenic lines, oak acetonic extractives and low lignin–low glucose, average glucose–average lignin conditions. Major intrinsic protein genes were expressed in nitrogen- and carbon-limited, ball-milled aspen, spruce wood 96-h incubated samples. ABC transporter genes were expressed in glucose, transgenic line 82, parent line P717, low lignin–high glucose, average lignin–average glucose, control, spruce wood 40-h incubated conditions. Major facilitator superfamily-coding genes were highly expressed in ball-milled aspen, carbon-, nitrogen-limited medium, high lignin–low glucose, transgenic line 64, 96-h incubation and control growth conditions. Tetra/Oligopeptide transporters were differentially expressed in nitrogen-limited medium, ball-milled aspen, low lignin–high glucose, average lignin–average glucose growth conditions. Fumaryl acetoacetase-encoding genes were expressed in high lignin–low glucose; fumarate reductase gene was expressed in spruce wood 40-h incubation samples. Short-chain dehydrogenase/reductase-encoding genes were expressed in carbon- and nitrogen-limited, spruce wood 96-h samples, control samples (GSE54542), transgenic line 64, low lignin–high glucose, average lignin–average glucose samples. Aromatic ring hydroxylase-encoding genes are differentially expressed in cellulose, spruce wood 40 h of incubation, average lignin–average glucose, low lignin–high glucose conditions, transgenic line 64 growth conditions. Dienelactone hydrolase-coding genes are highly expressed in glucose, ball-milled aspen, and average lignin–average glucose conditions. Due to their little reactivity, aromatic compounds derived from lignin degradation are usually attacked with the help of oxygen by oxygenases which results in intermediates like catechol or protocatechuate (Dagley et al. 1960; Fuchs et al. 2011). Mainly the process of peripheral degradation is commenced through central ring cleavage which is oxidatively catalyzed by ring cleavage dioxygenases (Gibson and Parales 2000; Vaillancourt et al. 2006). Extradiol ring hydroxylase genes are highly expressed in parent line P717, transgenic line 82, intradiol ring cleavage dioxygenase is highly expressed in high lignin–low glucose conditions.

Aldehyde dehydrogenase-encoding genes were differentially expressed in glucose, replete, nitrogen-limited, ball-milled aspen, transgenic line 64, low lignin–high glucose and spruce wood 96-h incubated conditions. Zinc alcohol dehydrogenase-encoding genes were highly expressed in replete, ball-milled aspen, transgenic line 64, low lignin–high glucose (20 days) growth conditions. Aldo/keto reductase-encoding genes were highly expressed in glucose, carbon-limited, transgenic line 64. According to Robson et al., aldo/keto reductases support the process of lignin degradation by providing hydrogen peroxide and ROS through oxidation of NADPH (Tramontina et al. 2017). Beta keto acyl synthase gene is highly expressed in glucose, ball-milled aspen, low lignin–high glucose, average lignin–average glucose growth conditions. Acetamidase or formamidase is expressed in nitrogen-limited, low lignin–high glucose, average lignin–average glucose conditions. It was reported that genes encoding for enzymes acetamidase, formamidase, uricase and amidohydrolase were found to be highly expressed by *P. chrysosporium* during nitrogen limitations (Tonon et al. 1990). Acetate kinase gene was expressed in replete, ball-milled aspen conditions, earlier studies have reported that metabolism of hydroxycinnamic acids (vanillin, *p-coumaric* and ferrulic acids) in *Streptomyces setonii* resulted in accumulation of acetic acid by causing shift in activities of alcohol dehydrogenase to acetate kinase (Filannino et al. 2014; Sutherland et al. 1983). Taurine catabolic dioxygenase is highly expressed in ball-milled aspen growth condition; 3-hydroxyacyl-CoA dehydrogenase gene is highly expressed in glucose medium. Genes coding for 2-nitropropane dioxygenase

were highly expressed in low lignin–high glucose, average lignin average glucose conditions. Carbonic anhydrase genes were highly expressed in transgenic line 64 and average lignin–average glucose conditions.

Haloacid dehalogenase genes were highly expressed in transgenic line 82, average lignin–average glucose and ball-milled aspen conditions. Phenylalanine ammonia lyase-encoding genes were highly expressed in nitrogen- and carbon-limited growth mediums, ball-milled aspen, transgenic line 64, oak acetonic extracts, average lignin–average glucose growth conditions. Homogentisate 1,2-dioxygenase-encoding genes were highly expressed in control, transgenic line 64 conditions and high gene expression values were observed for ball-milled aspen, replete, carbon-, nitrogen-limited growth mediums. Generic methyltransferases (or) *O*-methyltransferase-encoding genes were highly up-regulated in average lignin–average glucose, low lignin–high glucose, spruce wood 96-h incubated and replete samples. Gene expression studies conducted earlier have proved that lignin and its derivatives induce the higher expression of cytochrome P450 monooxygenase-encoding genes which are also involved in further degradation of lignin and its derivatives (Anzenbacher and Anzenbacherova 2001; Doddapaneni and Yadav 2005; Subramanian and Yadav 2008). Studies have already proved that plant cell wall polymers lignin and hemicellulose occur in deacetylated forms; it was also reported that these polymers are linked with *p*-coumaric acid and ferulic acid which are deacetylated by carbohydrate esterases such as feruloyl esterases, respectively (Biely 2012; Pawar et al. 2013; Špániková and Biely 2006). Deacetylases belonging to carbohydrate esterase classes are of high significance as the acetylated plant cell wall polymers cease the action of microbial enzymes. Genes encoding carbohydrate esterase (CE) classes CE-4 and CE-9 were found to be highly expressed in natural plant biomass growth substrates in 40-h spruce wood samples and CE-1, CE-4, CE-8, CE-15 and CE-16 in 96-h spruce wood samples (Kameshwar and Qin 2017) (Table 2).

Detoxification and stress-responsive genes

During the process of initial infection, *P. chrysosporium* and other basidiomycetes fungi produce extracellular reactive oxygen species (ROS) which are involved in breakdown of lignin, while studies have reported that expression of lignin peroxidase is linked with ROS production (Thuillier et al. 2014). However, during the process of wood decay, fungi come across highly reactive and toxic plant extractives which also exhibit a strong antifungal activity. So, efficient ligninolytic fungi should also exhibit the strong capacity to resist from the anti-fungicidal properties of plant extractives and lignin derivatives (Thuillier et al. 2014). Thus, fungi have developed a significant detoxification system which majorly includes cytochrome P450 complex enzymes, glutathione-*S*-transferases (Thuillier et al. 2014). Gene expression studies especially GSE52922 and other growth substrates containing natural plant biomass growth mediums (GSE69461, GSE54542, GSE69008, GSE27941) have showed higher expression of various genes encoding for detoxification and stress-responsive enzymes. The cellular ROS level significantly influences the redox state controlled by the degree of oxidation/reduction of active redox species, further regulating the cellular metabolism among which pyridine nucleotides and thiol/disulfide compounds play a crucial role as they bridge enzymes of intricate metabolic networks (Bunik 2003). Genes coding for 2-oxo acid dehydrogenase were highly expressed in cellulose, nitrogen-limited, 64-transgenic line, average lignin–average glucose (10 day) growth mediums.

Table 2 Differentially classified lignin-degrading enzymes obtained from different gene expression datasets of *P. chrysosporium*

P. chrysosporium genes involved in lignin degradation	
Lignin-degrading enzymes (first level)	Lignin peroxidase, manganese peroxidase, glucose oxidase, glyoxal oxidase, benzoquinone reductase, amine oxidase, aryl alcohol oxidase, chloroperoxidase, copper radical oxidase, multicopper oxidase, pyranose-2-oxidase, copper amine oxidase, phenylalanine ammonia lyase
Aromatic compound degrading enzymes (second level)	Intradiol dioxygenases, extradiol dioxygenases, aromatic ring hydroxylase, homogentisate 1,2-dioxygenase, epoxide hydrolase, cytochrome p450 monooxygenase, alcohol dehydrogenase, dioxygenase, 2-nitropropane dioxygenase, acireductone dioxygenase, ferredoxin, flavin containing monooxygenase, iron reductases, fumarate reductase, catalase, alcohol/methanol oxidases, formate dehydrogenase, haloacid dehalogenase hydrolase, oxidoreductase, prenyl transferase /squalene oxidase, acetamidase, formamidase, uricase
Other necessary enzymes (tertiary level)	Esterase/lipase/thioesterase, GMC oxidoreductase, metallophosphoesterase, short-chain dehydrogenase/reductase, D-isomer-specific 2-hydroxyacid dehydrogenase, beta-ketoacyl synthase, beta lactamase, 2-oxo acid dehydrogenase, aldo/keto reductase, aldehyde dehydrogenase, alkyl hydroperoxide reductase, amidohydrolase, delta-1-pyrroline-5-carboxylate dehydrogenase, 2-hydroxyacid dehydrogenase, FAD-linked oxidase, thiolase, hydroxymethylglutaryl-CoA synthase, carbohydrate esterases, glycosyl transferases

2-Oxoglutarate dehydrogenase was highly expressed in 96-h spruce wood growth medium. Thioredoxin-encoding genes were highly expressed in carbon- and nitrogen-limited, BMA, oak acetonic extractives, P717, transgenic line 82, high lignin–low glucose, 40-h spruce wood growth samples, at the same time peroxiredoxin (ubiquitous group of antioxidant enzymes)-encoding genes were expressed in 96-h growth samples. Genes encoding thaumatin pathogenesis-related protein were highly expressed in BMA, low lignin–high glucose growth mediums. The constant rates of NAD(P)H/NAD(P)$^+$ and SH/S-S facilitate the redox reaction by directly effecting on the proteins (Bunik 2003). Alkylhydroperoxide reductase and thiol-specific antioxidant are involved in reducing the reduced dithiol form of organic hyperoxides and protect against sulfur-containing radicals; genes encoding these enzymes were differentially expressed in glucose, replete, BMA, P717, transgenic line 82 growth samples. The three-major cell damaging units such as hydrogen peroxide, ROS and superoxide dismutase (SOD) are efficiently tackled by the fungal cells, if not leads to strong toxic stress to the cell and completely damages the cellular material, enzymes pertaining to it are catalase, peroxiredoxin and superoxide dismutase. Genes encoding catalase and superoxide dismutase enzymes are BMA, replete, high lignin–low glucose growth mediums, and transgenic line 64, control, high glucose–low lignin growth samples, respectively. Heat-shock proteins (HSP) and ubiquitin conjugating systems were highly expressed gene systems among the *P. chrysosporium* detoxification and stress-responsive mechanisms. Heat-shock protein 20 (Hsp20)-encoding genes were highly expressed in replete, BMA, high glucose–low lignin, average lignin–average glucose growth conditions. Similarly, several ubiquitin conjugating enzymes and complexes were highly expressed in plant biomass growth conditions such as P717, transgenic line 64, high lignin–low glucose and average lignin–average glucose growth conditions. Abortive infection protein was found to be highly expressed in high lignin–low glucose, control and stress-responsive proteins were highly expressed in 40-h spruce wood samples. According to Robson et al., aldo/keto reductases are required for various metabolic reactions such as degradation of β-aryl ethers present in lignin, degradation of carbohydrates and detoxification of xenobiotic compounds (Tramontina et al. 2017) (Table 3).

Phase I metabolic enzymes

The biotransformation of xenobiotic compounds in fungal cells commences majorly through phase I and phase II reactions. The phase I metabolic reactions majorly include transformation of parent compound to polar metabolites through de novo formation of functional groups such as –OH, –NH_2, –SH (Anzenbacher and Anzenbacherova 2001; Jancova et al. 2010). Phase I metabolic enzymes such as cytochrome P450 monooxygenases, epoxide hydrolases and dioxygenases are involved in N- and O-dealkylation, hydroxylation of aliphatic and aromatic compounds, N- and S-oxidation and deamination reactions (Jancova et al. 2010). The ability to protect against harmful toxic external xenobiotic compounds can be majorly imparted to the complex enzymatic defense systems majorly including cytochrome P450 monooxygenases. In 2005, Doddapaneni and Yadav have performed a customized genome-wide microarray of *P. chrysosporium* for studying the global expression of cytochrome P450 monooxygenases under nutrient-rich and nutrient-limited growth conditions. Out of 150 cytochrome P450-encoding genes expressed, 23 genes were differentially expressed by 2.0- to 9.0-fold in nitrogen-rich conditions and four genes expressed by 2.0- to 20-fold in low-nitrogen conditions, respectively. Subramanian and Yadav (2009) have performed a genome-wide role of cytochrome P450 monooxygenases in nonylphenol degradation by *P. chrysosporium*. This study has revealed that nonylphenol has induced multiple P450 monooxygenases out of which 18 genes were expressed with a fold change of 2–195 in nutrient-rich conditions, in low-nutrient growth conditions 17 genes with fold change of 2–6 and three genes were found to be expressed common among both these conditions. The current metadata analysis study has revealed the expression of cytochrome P450 monooxygenase-encoding genes especially in natural plant biomass

Table 3 Differentially classified lignin-degrading enzymes obtained from different gene expression datasets of *P. chrysosporium*

P. chrysosporium cells genes involved in detoxification and stress-responsive pathways	
Detoxification and stress-responsive enzymes	2-Oxo acid dehydrogenase, alkylhydroperoxide reductase, peroxiredoxin, manganese/iron superoxide dismutase, amidohydrolase Beta lactamase, urease, isoflavone reductase, dihydroorotate dehydrogenase, abortive infection protein, heat-shock protein, ubiquitin enzyme complex, thaumatin, thioredoxin, ferredoxin, flavodoxin, thiolase, catalase, aldo/keto reductases, etc.
Phase I metabolic enzymes encoding genes	Cytochrome P450 monooxygenases, epoxide hydrolases
Phase II metabolic enzymes encoding genes	UDP-glucuronosyltransferases, sulfotransferases, *n*-acyl transferases, glutathione-*S*-transferases, thioredoxin

containing growth substrates. In GSE14736, cytochrome P450-encoding genes were found to be expressed in BMA (GSE14734, GSE27941), replete and nitrogen-limited conditions with twofold expression. In 52,922 dataset genes encoding cytochrome P450 monooxygenases were differentially expressed in 64 and 82 transgenic lines, respectively. In GSE54542, GSE69008 and GSE69461 datasets cytochrome P450-encoding genes were highly expressed in control, low lignin–high glucose, average lignin–average glucose and 40-h spruce wood growth samples. Epoxide hydrolases, one of the important phase I metabolic enzymes, are required for the cellular epoxide or oxiranes transformation. These enzymes majorly exhibit three functions: (a) detoxification, (b) catabolism and (c) regulation of signaling molecules (Morisseau and Hammock 2005). Oxiranes or epoxides are highly toxic compounds effecting the cellular growth and development, epoxide hydrolases released by fungal cells catalytically add water molecules to the epoxides by resulting in corresponding 1,2-diols or glycols (Morisseau and Hammock 2005; Oesch 1973). Genes encoding epoxide hydrolases were found to be highly expressed in 40-h spruce wood, high glucose–low lignin, average lignin–average glucose transgenic lines, control, BMA growth conditions. Based on the normalized values, epoxide hydrolase was found to be differentially expressed in transgenic line 82 and glucose growth samples.

Phase II metabolic enzymes

The phase II enzymes also play a crucial role in biotransformation of xenobiotic and endogenous compounds by inactivating the active substances and converting it to easily excretable forms (Jancova et al. 2010). Phase II enzymes majorly perform conjugating reactions by employing transferases such as glutathione-*S*-transferases, UDP-glucuronosyltransferases, sulfotransferases, GCN5-acyltransferases, *O*-methyltransferases, NAD(P)H quinone oxidoreductases, MAPEG (membrane-associated proteins in eicosanoid and glutathione metabolism) and GFA (glutathione-dependent formaldehyde-activating enzyme) (Jancova et al. 2010). As reported in our previous work glycosyl transferases belonging to GT-1, GT-2, GT-4, GT-8, GT-20, GT-35, GT-39 and GT-48 were found commonly expressed among various datasets. Glycosyl transferases were found to be highly up-regulated in growth mediums containing complex plant biomass. Genes encoding glycosyl transferases expressed among the datasets were BMA (GT-1, GT-2, GT-20 and GT-39), glucose and replete (GT-8, GT-48), oak acetonic extracts (GT-2), high lignin–low glucose (GT-1, GT-4, GT-20), low lignin–high glucose (GT-2, GT-8, GT-15, GT-35, GT-39, GT-48), average glucose–average lignin (GT-2, GT-8, GT-15, GT-20, GT-35, GT-39, GT-48), P717 hybrid line and transgenic line 82 (GT-39), 40-h spruce wood samples (GT-1, GT-2, GT-15, GT-48), respectively. Genes encoding GCN5-acyltransferase were highly expressed in carbon-limited and replete growth mediums, transgenic line 64, low lignin–high glucose, average lignin–average glucose and 96-h spruce wood samples. *O*-Methyltransferase-encoding genes were highly expressed in low lignin–high glucose, average lignin–average glucose, 40- and 96-h spruce wood samples, transgenic line 64, carbon- and nitrogen-limited, BMA, control growth samples. Glutathione-*S*-transferase-encoding genes were differentially expressed in glucose, nitrogen-limited, BMA, oak acetonic extracts, transgenic line 64, low lignin–high glucose and average lignin–average glucose growth conditions 96-h spruce wood samples. Other enzymes constituting the glutathione system, such as glutathione-dependent formaldehyde activator, were differentially expressed in control and 96-h spruce wood samples and high lignin–low glucose based on their expression values. MAPEG-encoding genes were expressed in transgenic line 64 and oak acetonic extract growth samples. Folate cycle plays a crucial role in the maintenance of glutathione levels by sequestering formaldehyde (a toxic compound) formed from endo- and exogenous compounds. Our present data analysis has revealed that genes encoding for methylenetetrahydrofolate reductase and dihydrofolate reductase enzymes were found commonly expressed by *P. chrysosporium* when cultured on complex plant biomass growth mediums. MTHFR-encoding gene was highly expressed in glucose, nitrogen-limited, low lignin–high glucose, average lignin–average glucose and 96-h spruce wood growth samples, DHFR-encoding gene was differentially expressed in high lignin–low glucose. In oak acetonic extract samples, tetrahydrofolate dehydrogenase-encoding gene was highly expressed. Thiolase-encoding gene was highly expressed in oak acetonic extract, replete, BMA and 40-h spruce wood samples.

Effect of growth substrate and incubation period

Based on the gene expression studies and present metadata analysis we have found that *P. chrysosporium* gene expression is strongly influenced by the growth substrate. The gene expression profiles of *P. chrysosporium* were significantly different when cultured on synthetic (such as glucose, cellulose, carbon-limited, nitrogen-limited and replete growth mediums) and natural plant biomass growth substrates (such as ball-milled aspen, ball-milled pine, oak acetonic extracts, natural and genetically modified *Poplar* wood substrates). When *P. chrysosporium* was cultured on simple synthetic growth substrates (glucose, cellulose and replete) genes encoding for CAZymes were highly expressed along with genes required for the normal cell progression, growth and metabolism were found to be

highly expressed. Interestingly, genes encoding for animal haem peroxidase and chloroperoxidase were down regulated in carbon limited, nitrogen limited, replete and high lignin and low glucose growth substrates, while the same genes were found to be up regulated in glucose, cellulose, ball milled aspen, high glucose–low lignin and average lignin and average glucose growth conditions (Fig. 1). At the same time, several other genes encoding for lignin-degrading enzymes such as lignin peroxidase, glyoxal oxidase, copper amine oxidase, flavin amine oxidase, cytochrome P450 monooxygenase, glycosyl transferases 2OG-Fe(II) oxygenase family and several other enzymes were up-regulated when cultured in carbon- and nitrogen-limited growth mediums. When *P. chrysosporium* was cultured in natural plant biomass growth substrates, various genes encoding aromatic compound-degrading enzymes were highly expressed (Table 2). Genes encoding stress and detoxification-responsive enzymes were found to be highly expressed in these datasets, as natural plant biomass substrates also contain different plant extractives like flavonoids, tannins, quinones and stilbenes which cause severe toxicity to the fungal cells. Apart from the toxic plant extractives lignin and its degraded products also induce toxicity and stress on the fungal cells, due to which several stress-responsive genes were highly expressed by the fungal cells such as cytochrome P450 monooxygenases, glutathione-*S*-transferase, thaumatin, abortive infection, heat-shock and ubiquitin complex proteins. Genes involved in DNA, RNA and protein modification especially proteases (serine/threonine, aspartic) were also found to be highly expressed in natural plant biomass growth substrates.

The gene expression pattern of *P. chrysosporium* is also significantly influenced by the incubation period, gene expression studies especially GSE69008, GSE69461 and

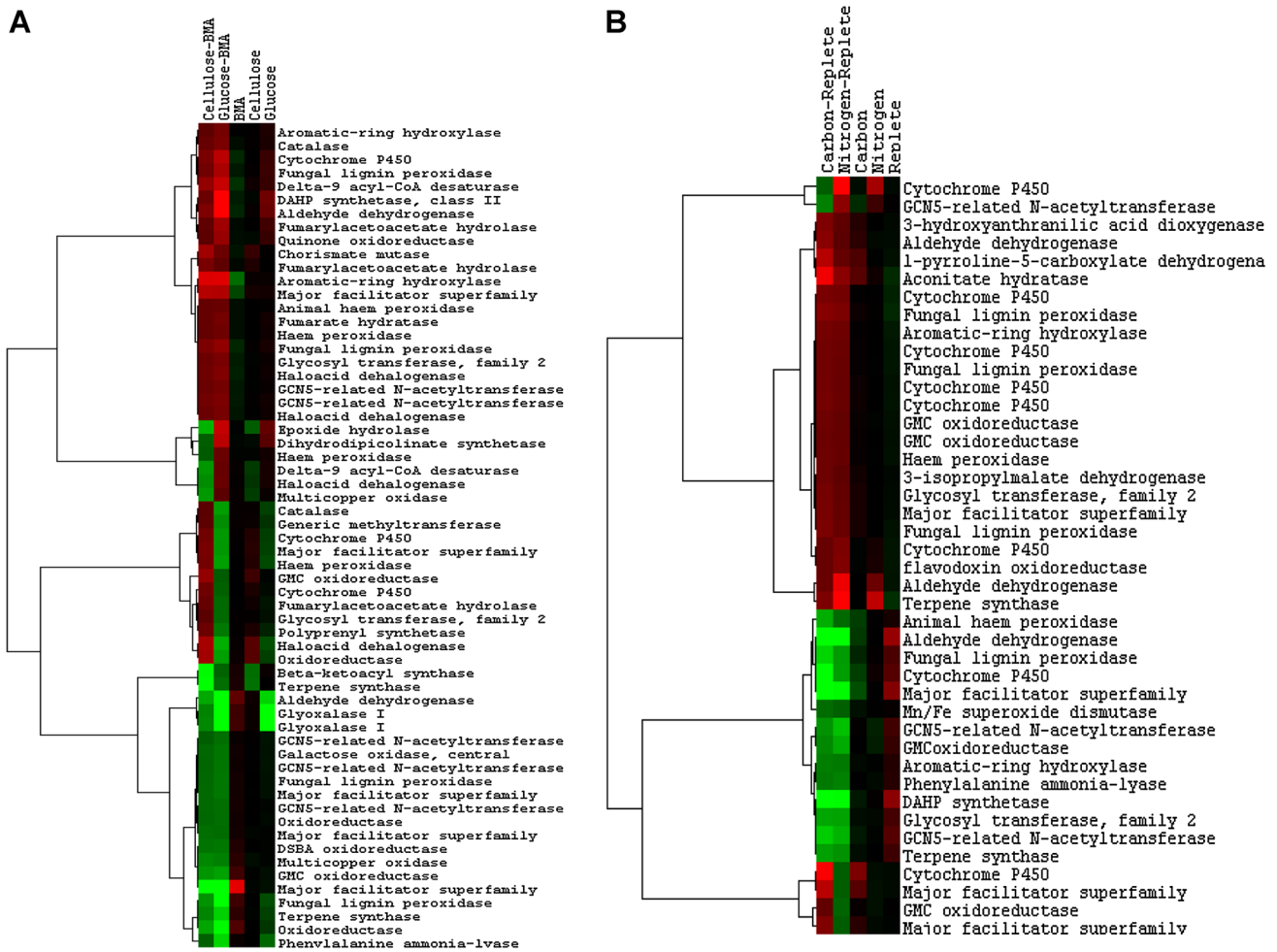

Fig. 1 Hierarchical clusters showing the differentially expressed genes obtained, 1st and 2nd columns list the fold change expression values up-regulated in cellulose and glucose (**a**) GSE14734, carbon and nitrogen limited in (**b**) GSE14735 and 3rd, 4th and 5th columns in **a** and **b** list the log-transformed gene expression values of BMA, cellulose, glucose, carbon-limited, nitrogen-limited and replete growth mediums, respectively

GSE6649 confirms it. Lignin being a large heterophenolic polymer requires a wide range of enzymes and complex systems for its degradation. In studies GSE69008, GSE69461 and GSE6649, *P. chrysosporium* gene expression was monitored for 10, 20, 30 days, 40 and 90 h and Day 2, Day 3, respectively. Results obtained from GSE69461 dataset show that genes encoding for lignin and manganese, chloro peroxidases, alcohol oxidase, amine oxidases, multicopper oxidases, phenyl ammonia lyase (forming lignin-degrading enzyme system) were highly expressed in 90-h incubation periods. Whereas in 40-h incubation period samples genes encoding for cytochrome P450 monooxygenase, fumarate reductase, aryl alcohol dehydrogenase, alternative oxidase, copper radical oxidase, FAD binding, phenol 1,2 monooxygenases were highly expressed. In GSE69008 dataset gene expression was significantly influenced by both the substrate and incubation period. The genes encoding for lignin degradation and genes involved in detoxification responses were also expressed among the 10- and 20-day incubation period samples (Fig. 2).

Discussion

The complex and stable structure of lignin makes its degradation process slow, except few aerobic fungi, aerobic and anaerobic bacteria even plants lack the metabolic pathways required for recycling lignin (Fuchs et al. 2011).

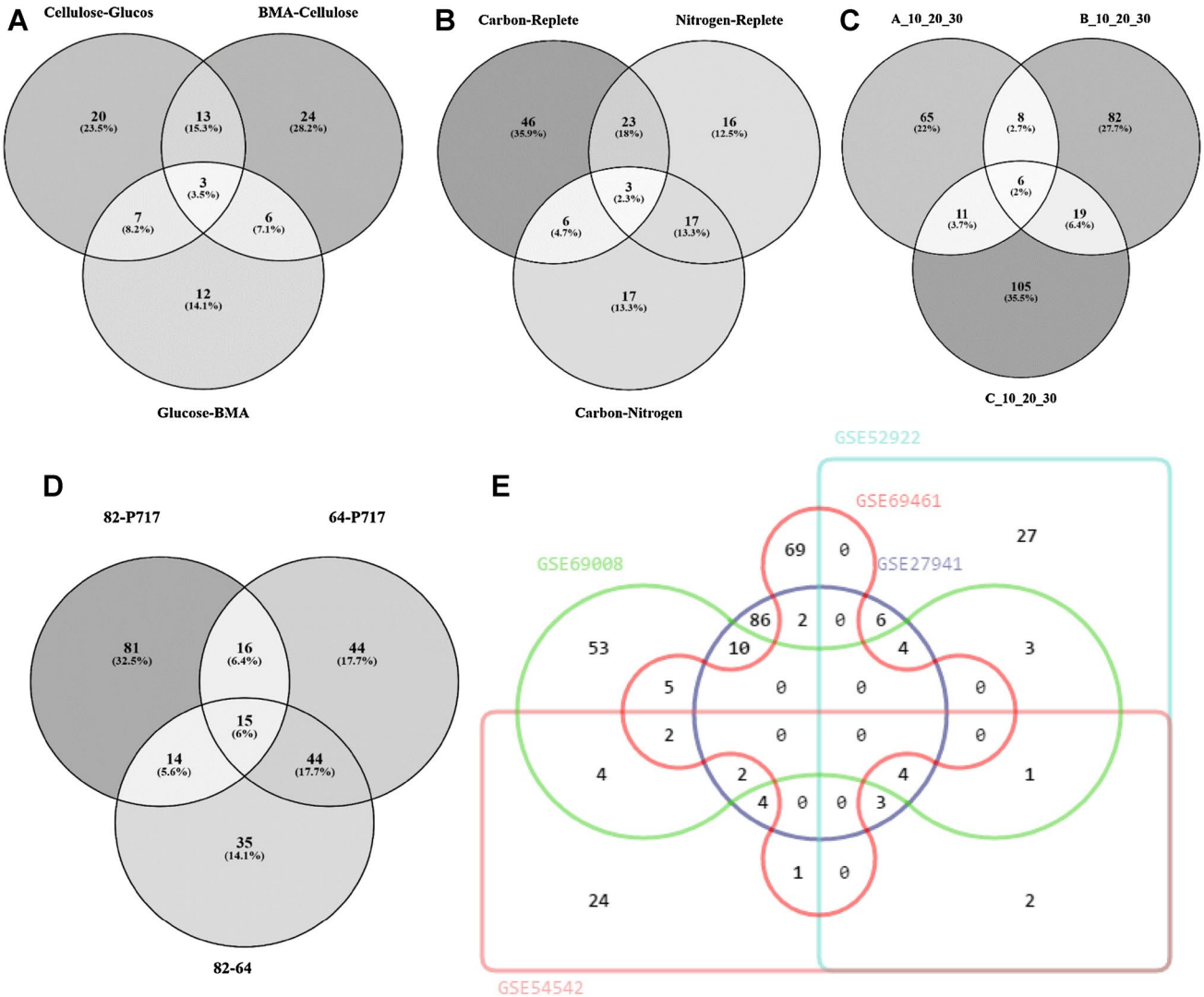

Fig. 2 Venn diagrams of differentially expressed genes obtained from the gene expression datasets. **a** GSE14734, **b** GSE14735, **c** GSE69008 (where **a, b, c** represent high lignin–low glucose, high glucose–low lignin, average lignin–average glucose, 10, 20, 30 represent number of days), **d** GSE52922 (where P717, 82 and 64 represent parent and transgenic lines of *Poplar trichocarpa* species, respectively), **e** GSE27941, GSE52922, GSE54542, GSE69008, GSE69461 (*Picea glauca* species)

Lignin-oxidizing enzymes of *P. chrysosporium* include lignin peroxidase (ten lip), manganese peroxidase (five mnp) and one (NoP/VP). These peroxidases are haem-containing proteins by containing a ferric heme group [Fe(III)] at its resting state which reacts with hydrogen peroxide (H_2O_2) resulting in compound-I oxo-ferryl intermediate (two-electron oxidized) containing [Fe(IV)]. The compound-I further oxidizes the donor substrate forming a second intermediate compound-II, both the reactions release free radicals (Bugg et al. 2011; Kameshwar and Qin 2016). The catalytic action of lignin peroxidase is aided by veratryl alcohol (diffusible oxidant) produced by *P. chrysosporium* (as a metabolite) VA cation radical reacts with lignin molecule on remote locations. VA also supports in functioning and higher expression of LiP enzyme (Jensen et al. 1994; Kameshwar and Qin 2016). Veratryl alcohol is produced by phenylalanine ammonia lyase (PAL) which catalyzes the first step in the VA formation by oxidizing L-phenylalanine to cinnamic acid and free ammonium ion through non-oxidative deamination (Hyun et al. 2011). Another enzyme *O*-methyltransferase transfers two-ring methoxyl group on VA (Korripally et al. 2015). Whereas Mn(II) being highly oxidant which acts as diffusible oxidant reacts with the lignin molecule even on the remote locations of lignin without the catalytic active center getting involved through oxidizing the lipid peroxidation reactions wherein delta-9 fatty acid desaturases support the catalytic function of MnP (Bugg et al. 2011).

Ligninolytic peroxidases are similar to classic peroxidases in their function as they are dependent on hydrogen peroxide for their function. The process of hydrogen peroxide generation in *P. chrysosporium* is controlled by set of enzymes glyoxal oxidase, pyranose oxidase, aryl alcohol oxidase, veratryl alcohol oxidase, amine (copper amine) copper radical oxidase and alcohol oxidases. Genes encoding glyoxal oxidase, copper amine oxidase, alcohol oxidase, copper radical oxidase were commonly expressed among various datasets as explained above. Two unconventional genes encoding for chloroperoxidase, animal haem peroxidase were also being highly expressed on cellulose, BMA, replete, transgenic line 64 and low lignin–average glucose growth substrates. Based on the expression of genes coding for ferric reductase, ferroxidase, cellobiose dehydrogenase, LPMO and quinone reductase in plant biomass containing growth substrates, it is proposed that *P. chrysosporium* depends on these enzymes for generation of toxic hydroxy radicals and in iron homeostasis. The hydroxy radicals generated upon reaction of hydrogen peroxide and iron-oxalate complex, these highly toxic radicals (OH*) attack the complex lignin molecules (Bugg et al. 2011). The above-mentioned enzymes can be classified to participate in primary ligninolytic reactions based on their functional properties and due to their common expression among all the growth substrates (Fig. 3). Along with the above-mentioned lignin-oxidizing enzymes, a wide range of aromatic compound-degrading enzymes, stress-responsive and detoxifying enzymes operate in coordination to degrade lignin and its derived products.

Degradation of lignin molecules by *P. chrysosporium* results in various low molecular weight chemical compounds, if progressive and controlled lignin breakdown strategies are developed it can lead to the production

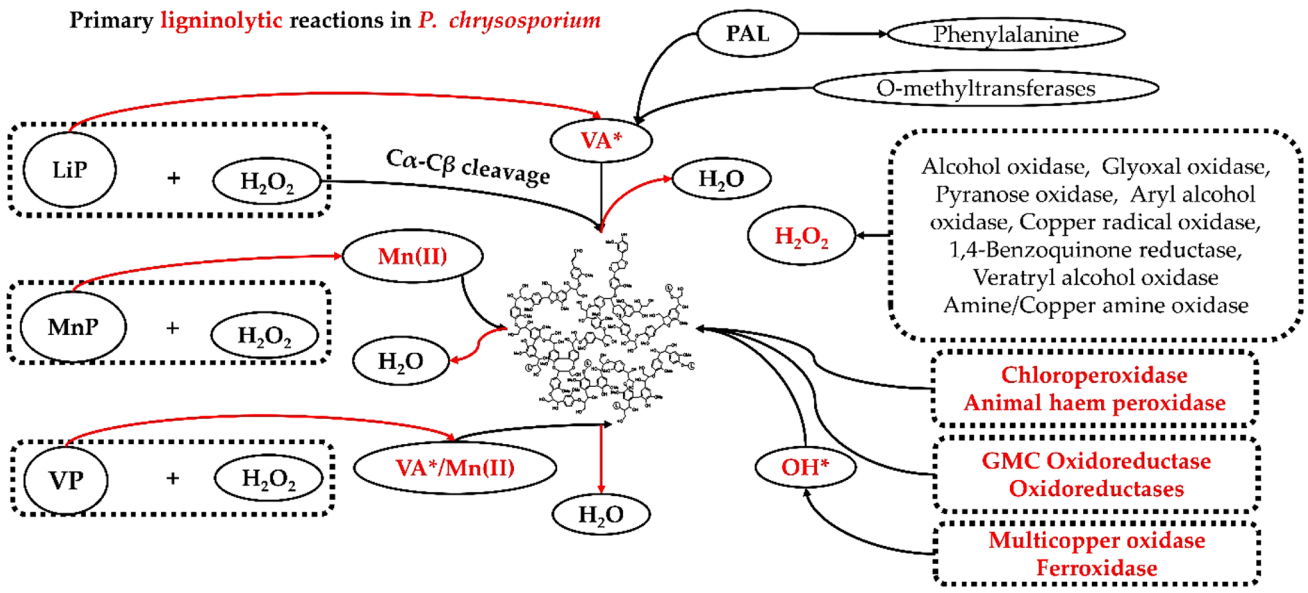

Fig. 3 The primary enzymatic reactions of *P. chrysosporium* involved in lignin degradation: LiP (lignin peroxidase), MnP (Manganese peroxidase), VP (Versatile peroxidase), VA (Veratryl Alcohol), PAL (Phenylalanine ammonia lyase), MCO (Multicopper oxidase)

useful renewable and green platform chemicals (Bugg et al. 2011). Studies conducted previously on spruce wood degradation by *P. chrysosporium* has resulted in total 28 low molecular weight chemical compounds out of which 10 were aromatic carboxylic acids (Chen et al. 1982) and 13 were acyclic 2,4-hexadiene-1,6-dioic acids obtained through oxidative ring cleavage (Chen et al. 1983). The chemical compounds obtained from lignin degradation were derivatives of benzoic acids by oxidative cleavage of Cα–Cβ of lignin components. The biphenyl and diphenyl ether dicarboxylic acids obtained were derivatives of biphenyl and diphenyl ether components of lignin (Bugg et al. 2011). Studies have also reported that metabolism of β-aryl ether model compound by *P. chrysosporium* involves Cα-Cβ oxidative cleavage resulting in vanillin (Enoki et al. 1980). Lignin peroxidase of *P. chrysosporium* catalyzes the oxidative cleavage of Cα–Cβ bond of various lignin derivative compounds (Kirk and Farrell 1987) such as diarylpropane (Enoki and Gold 1982), β-aryl ether model (Enoki et al. 1980), phenylcoumarane (Nakatsubo et al. 1981) compounds resulting in aromatic aldehyde products, vanillin compounds, respectively. It is well known that lignin peroxidase is also involved in degradation of non-phenolic units of lignin polymer (which constitutes to 90% of lignin polymer) at the same time MnP is involved in degradation of both phenolic and non-phenolic units of lignin compound (Bugg et al. 2011; Kameshwar and Qin 2016). According to Nakatsubo et al. (1981), the degradation process of alkylated phenylcoumarane by *P. chrysosporium* is directed via primary oxidation of side chains followed by the oxidation of heterocyclic ring to furan and then performs the oxidative cleavage of Cα–Cβ bond (Nakatsubo et al. 1981). The process of lignin degradation results in a wide range of aromatic compounds which are further degraded by a set of aromatic compound-degrading enzymes. Enzyme systems involving oxygenases attack these aromatic compounds in the presence of oxygen resulting in two central intermediate compounds catechol and protocatechuate which are further degraded by intra- and extradiol dioxygenases which catalyze the oxidative cleavage of central ring (Fuchs et al. 2011). Microorganisms employ variety of metabolic pathways divided as upper pathways (resulting in catechol and protocatechuate) and lower pathways (acetyl-CoA, succinyl-CoA and pyruvate) to efficiently utilize wide range of aromatic substrates generated due to the process of degradation (Fig. 4) (Fuchs et al. 2011). The upregulation of genes encoding for central ring cleaving dioxygenases (intra and extradiol dioxygenase), cytochrome P450 monooxygenases, aromatic ring hydroxylase dioxygenases, catechol 1,2-dioxygenase and other dioxygenases in complex plant biomass growth substrates supports the involvement of upper and lower pathways by *P. chrysosporium*.

Naturally, plant cell wall components especially hemicellulose and lignin occur in acetylated forms to cease the activity of various hydrolyzing enzymes of microbial origin (Pawar et al. 2013). Higher expression of genes encoding for carbohydrate esterases endorses earlier reports on deacetylation of lignin units. According to Del Rio et al. (2007) in lignin, acetyl groups were found to be associated with gamma carbon of aliphatic side chains situated on the syringyl and guaiacyl monomers of lignin with highest degree of acetylation observed in jute fibers, abaca and kenaf with 0.8 DA, acetylation of lignin in hardwood varies between the range of 1 and 50% (w/w) (Del Río et al. 2007). Along with carbohydrate esterases genes encoding for various glycosyl transferase class enzymes were found to be highly expressed in both customized synthetic and complex natural plant biomass growth substrates suggesting their role in controlling cellular toxicity. During the process of plant biomass degradation *P. chrysosporium* reacts with the toxic properties of lignin and its derivatives, thus there might be potential involvement of glycosyl transferases in changing the toxic properties of lignin derivative compounds. According to Le Roy et al. (2016), glycosyl transferases are significantly involved in detoxification of plants during the secondary metabolite synthesis (flavonoids, lignin and phenylpropanoids) and process of glycosylation modifies their solubility, stability and toxicity (Le Roy et al. 2016). In our analysis, we have also found that several genes involved in the process of DNA synthesis, modification and repair were highly expressed along with a set of transcription factors among the top differentially expressed genes. Genes encoding for various proteases especially serine, cysteine carboxyproteases were found to be commonly and differentially expressed among all the datasets (Fig. 5).

As discussed earlier, fungal cells are exposed to highly toxic and stressful environments; during the process of degradation fungal cells encounter polyphenolic lignin units and its degraded products along with other wood extractives (Nascimento et al. 2013; Shalaby and Horwitz 2015). It was also reported that various chemical bioproducts such as aldehydes, aliphatic acids, phenolic and furan derivatives are obtained because of lignocellulosic biotreatment methods which majorly inhibit the action of ligninolytic enzymes and further microbial fermentation (Ko et al. 2015). To protect from these toxic substances (degraded products of lignin and its derivatives, plant extractives and ROS, hydroxy radicals and superoxides/free radicals) *P. chrysosporium* secretes a wide range of antioxidant and stress-responsive enzymes such as cytochrome P450 monooxygenases, glutathione-*S*-transferases, catalases, superoxide dismutases majorly classified as phase I and

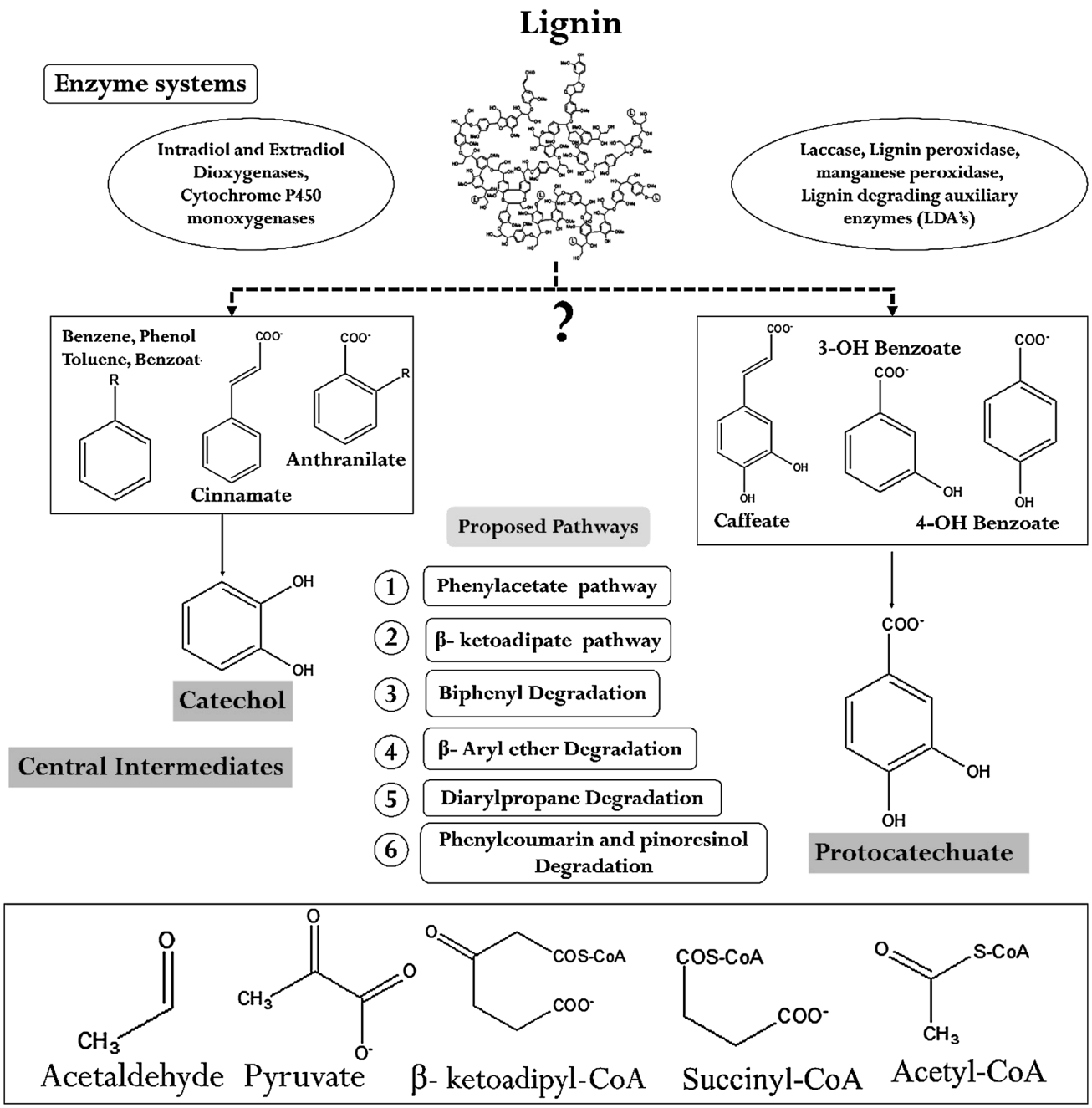

Fig. 4 Bird's eye view of tentative and proposed general molecular mechanisms and pathways involved in lignin degradation

phase II metabolic systems. We have clearly observed the differential expression of genes encoding for phase I and phase II enzyme systems by *P. chrysosporium* when cultured on complex plant biomass growth substrates (Fig. 6). The process of lignin degradation in *P. chrysosporium* is associated with ROS production, reactive oxygen and hydroxyl radicals which also cause additional toxicity. Results obtained from our present study and gene expression studies reported earlier strictly convey that the process of lignin degradation is interdependent on the intracellular detoxification systems and cellular redox states. Cellular redox states were characterized by redox-active species (such as thiol disulfide and pyridine nucleotide compounds) and their degree of oxidation/reduction reactions (Bunik 2003). While the balanced ratios of NADPH/$NADP^+$ and SH/S-S facilitate the process of redox regulation by effecting the proteins directly, at the same time NADPH/$NADP^+$ and SH/S-S ratios are directly related to the cellular ROS level (Bunik 2003). Genes coding for 2-oxoacid dehydrogenase multienzyme complexes which play a crucial role in

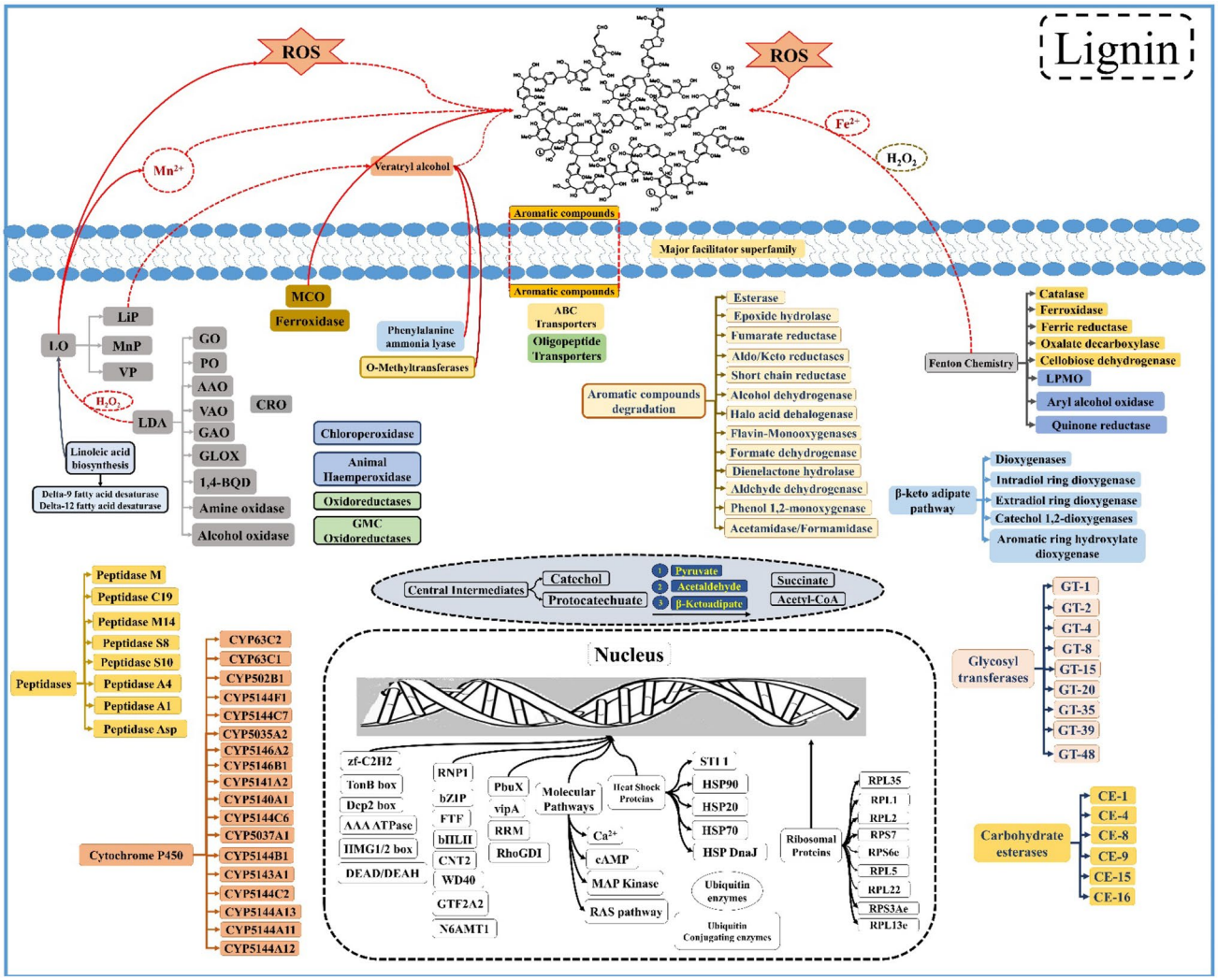

Fig. 5 Tentative network of *P. chrysosporium* genes and enzymes involved in lignin degradation mechanisms

regulating the cellular redox states were found to be highly expressed on complex natural plant biomass-based growth substrates. Similarly, higher expression of thioredoxin, glutathione-*S*-transferases, peroxiredoxin, NADPH oxidases, etc., explains that *P. chrysosporium* continuously maintains the cellular redox state and controls the toxic conditions developed due to lignin degradation. The present metadata analysis is in complete accordance with previous reportings made on production of ligninolytic enzyme machinery upon nitrogen repression and natural plant biomass containing growth substrates. We have also observed the expression of genes encoding for ligninolytic enzymes and intracellular antioxidant mechanisms simultaneously by *P. chrysosporium* when cultured on synthetic growth substrates mimicking ligninolytic conditions GSE6649, GSE14735 (nitrogen limited, carbon limited). Especially lignin and manganese peroxidases and lignin-degrading auxiliary enzymes were highly expressed in day 3 cultures and nitrogen-limited mediums of GSE6649 and GSE14735 datasets, respectively, followed by expression of enzymes such as cytochrome P450, thioredoxin, Mn^{2+}superoxide dismutase and other intracellular antioxidant enzymes supports the above process of degradation. Similar gene expression patterns were observed in *P. chrysosporium* when cultured on natural plant biomass growth substrates with clear expression of various enzymes involved in lignin degrading and detoxification, stress-responsive mechanisms.

Through our present analysis, we have demonstrated the functional involvement of various enzymes in lignin degradation and detoxification by *P. chrysosporium*. These outcomes clearly indicate that unlike cellulose, hemicellulose *P. chrysosporium* invests high proportions of molecular and metabolic systems in the process of lignin degradation. Based on the results obtained we

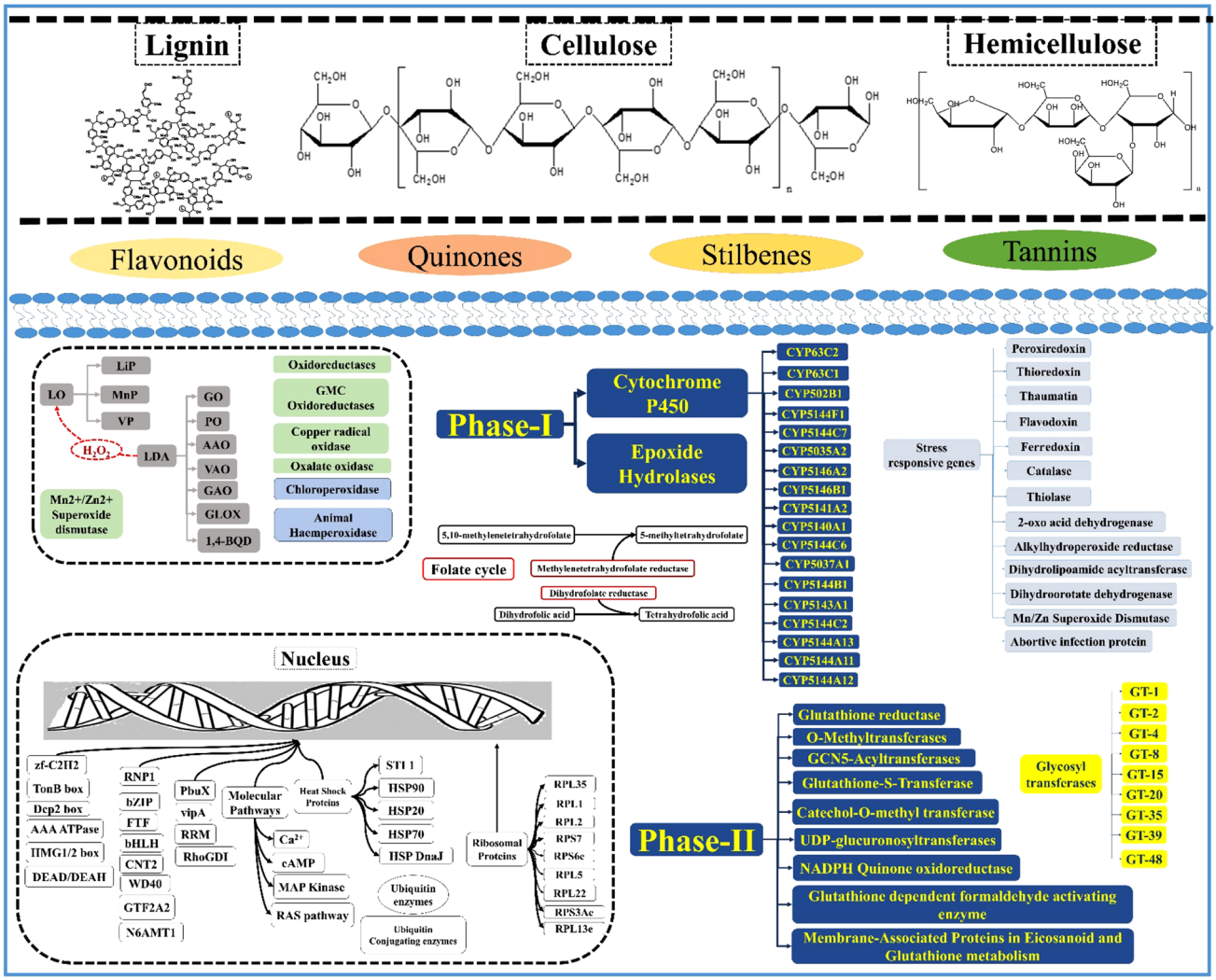

Fig. 6 Tentative network of *P. chrysosporium* genes and enzymes involved in detoxification mechanisms involving phase I, phase II and stress-responsive pathways

propose that process of lignin degradation in *P. chrysosporium* commences through synchronous expression of both ligninolytic enzymes and detoxification-stress-responsive systems. Both previous reports and present results convey that *P. chrysosporium* involves highly toxic ROS, free radicals, etc., along with conventional ligninolytic enzymes to create random cuts in the lignin structure, by creating the platform for detoxification and stress-responsive enzymes. However, there is certain ambiguity related with lignin degradation and detoxification-stress-responsive pathways proposed, as we have observed that several highly expressed genes were annotated as uncharacterized proteins. Further proteomic studies must be continued to understand their involvement in lignin degradation and detoxification-stress-responsive mechanisms of *P. chrysosporium*.

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Compliance with ethical standards

Conflict of interest The authors have declared that no competing interest exists.

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