Copper ion altered association network among multi-genes and enzyme activity of laccase in *Ganoderma lucidum*

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Background: Laccases, copper-based polyphenol oxidases, played vital roles in lignin and humus degradation as well as fruiting body formation and stress response. Sixteen putative laccase genes (*Lacc1-Lacc16*) were reported in the genome of white-rot fungus *Ganoderma lucidum*. Members in this multi-gene family usually had close inter-relationships and may vary in the roles contributing to functions. Identifying the interactions among multiple genes and thus the conjoined consequence to an activity was essential for systematically unraveling the molecular mechanisms of laccase and improving laccase activity.

Methods: In this study, multivariate statistical analysis was applied to track the relationship between thetranscriptional level of laccase genes and the total enzymatic activities. We outlined and compared the interaction networks among the transcriptional levels of 16 laccase genes and associations with the total enzymatic activities with or without copper ion (Cu^{2+}).

Results: A multi-gene interaction network among the sixteen genes and laccase activity was constructed to figure out the changes induced by Cu^{2+}. The interaction network showed that the enzyme activity was the result of interactions among genes, and these interactions might vary with the presence of Cu^{2+}, subsequently leading to the alteration of enzyme activity. Some genes always kept relation with enzyme activity (positive or negative, *Lacc13*, *Lacc10*), some were irrelevant (*Lacc1*, *Lacc6*), while another some were inconsistent (*Lacc3, Lacc8, Lacc14* and Lacc15).

Discussion: Network-based methods were applied to identify key functional genes and to outline associations among genes and phenotype in laccase multi-gene family. This is an exploratory strategy to describe the transcriptional complexity of laccase and its relevant responses to Cu^{2+} stress. The identified key functional genes associated with laccase activity (e.g. *Lacc10, Lacc13*) and the associations among genes and activity will benefit for the regulation of enzyme activity.

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Abstract

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Discussion: Network-based methods were applied to identify key functional genes and to outline associations among genes and phenotype in laccase multi-gene family. This is an

exploratory strategy to describe the transcriptional complexity of laccase and its relevant responses to Cu^{2+} stress. The identified key functional genes associated with laccase activity (e.g. *Lacc10, Lacc13*) and the associations among genes and activity will benefit for the regulation of enzyme activity.

Introduction

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2), a group of copper-based polyphenol oxidases, are among the most important extracellular enzymes secreted by white-rot fungi (1). Laccases catalyze the reduction of molecular oxygen to water by using a wide range of phenolic and aromatic compounds as hydrogen donors (2, 3, 4). Due to its substrate-broad and eco-friendly properties, laccases have been of great interests for potential industrial application, such as bioremediation (5, 6), dye decolorization (7, 8), food processing (9) and other applications (10). A total of 16 putative genes (NCBI accession no.: AHGX00000000) were identified in the genome of *Ganoderma lucidum*, one of white-rot fungi (11).

Laccases are encoded by a complex laccase multi-gene family (12). It is always diversified of the relationship among the genes in multi-gene family associated with the same phenotypes. Some may be in cooperation, some may be mutually exclusive, while others may be functionally redundant (13). Previous studies have often focused on the association between each single gene and phenotype (14), and have not been able to detect the combined effects of multiple genes. Whereas, the phenomenon that no obvious phenotypic alterations are observed in the morphology or biochemical parameters in single knock-out mutant (15) pointed out that missing activity could be compensated by a redundant enzyme, and functions are presented as the results of all gene-gene interactions. Therefore, investigating the interaction among genes and phenotypes is instructive to fully understand the molecular mechanism of a function and systematically regulate the expression.

In laccase multi-gene family, genes often have close relationship with various functions,

such as morphogenesis, stress defense, lignin degradation, etc. However, their interaction in degrading lignocellulose is still unclear. Copper (Cu), a co-factor for various enzymes, not only is an essential trace element for most living organisms, but also could stimulate laccase transcription and secretion (16, 17, 18). The details of laccase gene transcription for laccase secretion and its relevant responses to Cu^{2+} are still obscure.

In this study, we characterized the sixteen putative laccase coding genes in *G. lucidum* genome *in silico,* investigated the expression levels of these genes and total laccase activities at different growth time points with the presence or absence of Cu^{2+}. Multivariate statistical analysis methods including PCA (principal components analysis), PLS (partial least squares) and correlation analysis were applied to track the relationship between the transcriptional level of laccase genes and total laccase activity and to find important genes contributing to the total laccase activity. Gene-activity network based on the Kendall's Tau correlation was then constructed to compare the interaction differences induced by Cu^{2+} among the genes and the activity. This analysis elucidated the sixteen putative laccase genes' expression characteristics and their contributions to laccase activity during the growth of *G. lucidum*, as well as provided an exploratory strategy for identifying functional genes and for studying interactions of genes within a multi-gene family.

Materials & methods

Chemicals The 2,2'-azino-*bis* (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was purchased from Shanghai Hualan chemical technology co. LTD (China). Sodium acetate (NaAc), cetyl trimethyl ammonium bromide (CTAB) and compounds for media were of analytical grade

and from Sinopharm (China).

Strains and culture conditions. Strain P9 of *G. lucidum* (CCTCC AF 2014005 P5-9) was maintained on a potato-dextrose (2.4%, w/v) agar (PDA) medium for seven days at 25℃ and stored at 4℃. Agar plugs (diameter 6 mm) were obtained from the growing edge of a fungal colony growing on PDA plates and inoculated into 100 mL volumes of medium in a 250-mL flask. The liquid medium was revised based on Tien & Kirk's medium (19): straw powder (diameter 0.3 mm) 30 g/L, glucose 10 g/L, wheat bran extract 6 g/L, NH_4SO_4 0.005 g/L, basal medium 100 mL/L (basal medium: KH_2PO_4 0.2 g/L, $MgSO_4•7H_2O$ 0.05 g/L, $CaCl_2$ 0.01 g/L, VB_1 0.1 g/L, Tween-80 5 g/L), trace medium 1 mL/L (trace medium: $MnSO_4$ 0.5 g/L, $FeSO_4•7H_2O$ 0.1 g/L, $CoCl_2$ 0.1 g/L, $ZnSO_4•7H_2O$ 0.1 g/L, $CuSO_4•5H_2O$ 0.01 g/L, $AlK(SO_4)_2•12H_2O$ 0.01 g/L, H_3BO_3 0.01 g/L, $Na_2MoO_4•2H_2O$ 0.01 g/L). The fungal cultures were shaken at 150 r/min and kept at 25℃ in the dark. On the second day, $CuSO_4$ was added to the medium at the final concentration of 150 μmol/L in the Cu^{2+} group. The concentration of $CuSO_4$ and the time for $CuSO_4$ addition were obtained from our preliminary experiments. With the addition of $CuSO_4$ after 48 h incubation, not only did the fungus grow better but the activity of laccase was also higher. All experiments were performed at least three times by performing three replications for each treatment and each time point. For each treatment and its control, triplicate cultures were harvested from the second to the 14^{th} day, with laccase activity and total RNA isolation being measured in an interval of one day.

Enzyme activity determination. Extracellular laccase activities in supernatants were measured at 32℃ using ABTS as the substrate. The reaction mixture (1 mL) contained 0.1 mL of

0.1 mol/L NaAc buffer (pH 5.0), 0.8 mL of 0.03% (w/v) ABTS and 0.1 mL of culture supernatant. One enzyme unit (U) was defined as the amount of enzyme that oxidized 1 μmol ABTS per minute using an ε_{420} =3.6×10^4 mol^{-1} cm^{-1} (20).

RNA preparation and reverse transcription. The mycelium pellets were collected from each sample by centrifuging at 8000×*g* for 10 minutes. Mycelia were snap-frozen in liquid nitrogen immediately after sampling and stored at －80℃ for further use. Total RNA of *G. lucidum* was isolated using the modified CTAB method (21). RNA integrity was verified with agarose gel electrophoresis, and its purity and concentration were measured using the ultraviolet spectrophotometric method (NanoDrop-1000, USA). First strand complementary DNA (cDNA) was synthesized using the PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian, China).

Quantitative real-time PCR (qPCR). The gene-specific primers (Table S1) were designed with Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA). The glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) gene was chosen as an endogenous reference gene to normalize target gene expression. SYBR Premix Ex Taq II (TAKARA, Dalian, China) was used as reaction mixture, with the addition of 0.4 μL of each primer (10 μM), 1 μL of template cDNA and 3.2 μL of ddH_2O, with a final volume of 10 μL. qPCR was performed as follows (Bio-Rad CFX96 Real-Time PCR System, USA): 95℃ for 30 s and then 40 cycles of 95℃ for 5 s, 60℃ for 30 s. After amplification, the melting curves were generated in the range 65–95℃ with increments of 0.5℃ every 5 seconds to ensure the presence of a single amplicon. All experiments were conducted in triplicate and non-template controls were

131 performed to check for any potential contamination. Relative amounts of each transcript
132 were determined by $2^{-\Delta CT}$ ($\Delta C_T = C_{T\ target} - C_{T\ GAPDH}$), normalized with respect to *GAPDH* (22).

133 **Statistical analysis and network construction.** Principal components analysis (PCA),
134 partial least squares (PLS) regression combined with Martens' uncertainty test for important
135 variable detection (23), calculation of Pearson correlation, Kendall Tau rank correlation
136 coefficient and the Kendall correlation distance for network construction were all performed
137 using Matlab (The MathWorks, Natick, MA, USA) with build-in or in-house programs. The
138 gene-activity network was visualized with Cytoscape 2.6.0 (24). Only correlations with an
139 absolute value of 0.6 or greater were shown.

140 **Nucleotide sequence accession number.** The nucleotide sequences of the 16 laccase genes
141 were deposited in the NCBI database under the accession number AHGX00000000.

142 **Results**

143 **Laccase activity assay.** A laccase activity assay was conducted at different time points in *G.*
144 *lucidum* with or without Cu^{2+}. The dynamic curve of laccase activity revealed a stimulating
145 effect of Cu^{2+} supplementation on laccase secretion with a prolonged and higher enzymatic
146 activity peak (Fig. 1). This effect was observed throughout the entire experiment; the laccase
147 activity on the fourth day was especially enhanced to 1.52-fold, up to 148 U/mL, and on the sixth
148 day, it was elevated 1.86-fold, up to 165 U/mL, compared with the control.

149 **Gene expression and statistical analysis.** The gene expression profiles with or without
150 Cu^{2+} from the 4^{th} day to the 14^{th} day were quantitatively detected with qPCR using gene-specific
151 primers (Table S1). Time series monitoring of the control and Cu^{2+} groups indicated that all 16

laccase genes had their own expression patterns throughout the time course and also varied in different conditions (Fig. S1). The high percentage of genes with altered transcriptional responses to Cu^{2+} revealed a complex regulation mechanism that may be related to the sensitivity of the laccase gene family to Cu^{2+}. Some transcripts, including *Lacc4*, *Lacc7*, *Lacc11*, *Lacc14* and *Lacc16,* were minimally expressed in both the control and Cu^{2+} groups, whereas *Lacc8* was the most active gene in that it expressed at every developmental time point. The transcription level of *Lacc8* was not only higher than that of the other genes in the control condition but also increased to 2.97-fold with Cu^{2+}.

PCA, an unsupervised multivariate analysis method, was applied to track and compare the changes of the gene composition structures of the two groups with time. As shown in Fig. 2, by day four, the gene composition structures of the two groups differed and followed dissimilar time trajectories but ended at almost the same location on the 14^{th} day, suggesting similar gene compositions by this time point. On the 14^{th} day, both groups had the lowest extracellular laccase activities in their time courses and there was no significant difference between the two values. Of note, the gene compositions of the control group on the sixth and eighth days were very similar, but their laccase activities differed nearly 4-fold (17.38 U/mL, 68.16 U/mL, respectively). It supposed that the increased extracellular enzymatic activity on the sixth day was due to some laccase genes with low concentrations or by other undetected enzymes.

Though we could not rule out the possibility of the existence of other undetected enzymes, PLS regression models were used to describe the total extracellular laccase activity with the 16 measured gene expression levels, with the assumption that the majority of the total extracellular

laccase activity was from these 16 genes. PLS is a useful multivariate calibration method commonly used to determine a relationship between the predictors X and the response Y (25). The combination of PLS with Martern's uncertainty test could determine important X variables contributing to Y (23, 26). A global PLS model with six PLS components using two group samples yielded the best modeling result, i.e., the minimal cross-validated prediction error.

The Pearson correlation of the predicted activities with the real activities was 0.68 (p=0.01). Martern's uncertainty test based on the established PLS model did not find any important variables contributing to the total laccase activity at the p=0.05 level, but did find *Lacc13* to be important at the p=0.1 level. The highly positive significant correlation of *Lacc13* with the total laccase activity was further confirmed by Kendall correlation analysis (r=0.6, p=0.136 in the control group, r=0.733, p=0.056 in the Cu^{2+} group).

By monitoring the change of *Lacc13* with the total extracellular laccase activity in both groups, we determined that the higher the concentration of *Lacc13*, the higher the activity, except on the sixth day in the Cu^{2+} group (as indicated by an arrow in Fig. 3). On the sixth day, the concentration of *Lacc13* of the Cu^{2+} group was not high, but the activity was the highest (165.04 U/mL), again suggesting the possibility of activity contribution might come from other genes.

Kendall tau correlation analysis is a non-parametric method for measuring the strength of bivariate relationships using ranked scores (27, 28). The method is resistant to outliers, can measure both linear and nonlinear monotonic correlations and give accurate p-value even for a small sample size (29). Here, for our rather small data set, we applied the Kendall rank correlation coefficient to evaluate the association between two genes or gene-laccase activity

obtained at series time points. However, to evaluate all possible correlations, the pre-screen criteria was set to r≥0.6, with the worst p-value at 0.136. A network based on these correlations (Fig. 4) was then constructed to view and compare the global interactions among the genes and laccase activity in two groups. As shown in Fig.4, laccase activity was resulted from multiple genes and their interaction, and its formed gene-activity network was obvious dissimilar between the control group and the Cu^{2+} group. In the control group, *Lacc8*, *Lacc10*, *Lacc13*, *Lacc14* and *Lacc15* directly related with enzyme activity with *Lacc13*, *Lacc14* and *Lacc15* as the key genes. Most of the other genes which connected to laccase activity should relate with these key genes first. In the Cu^{2+} group, *Lacc3, Lacc10* and *Lacc13* showed its direct association with laccase activity as key genes. Regardless of Cu^{2+} presence, *Lacc13* always directly and positively contributed to enzymatic activity, which agreed with the above analysis. *Lacc10* showed a directly negative relation, whereas *Lacc1* and *Lacc6* were irrelevant to laccase activity.

Except the four genes (*Lacc1, Lacc6, Lacc10, Lacc13*), most of the other genes showed inconsistent associations with the laccase activity when the conditions had been changed. Some genes were directly connected with activity in one group with indirect or no connection in the other group (*Lacc3, Lacc8, Lacc14, Lacc15*), while some genes were irrelevant to laccase activity in one group with indirect association in the other group (*Lacc2, Lacc5, Lacc7, Lacc11, Lacc12* and *Lacc16*).

Discussion

Laccase is the most effective extracellular ligninolytic enzymes which could be used for the production of high valued compounds from lignin. The multiplicity of laccase genes and the

effect of copper on the laccase production and gene transcription have been observed (30, 31). However, little work has been done to elucidate the laccase gene interaction network and to link it to laccase activity or lignin-degrading ability under copper stress. Based on laccase gene expression profiles, we constructed an overall laccase gene-activity interaction network under the condition with or without Cu^{2+}.

The activity of laccase was considered as the results of interaction of a network of genes. One disturbed gene could be compensated by the other genes. It could explain why organism rarely experience global breakdown despite frequent routine problems. This laccase gene-activity network revealed some important or key genes directly related with laccase activity, while some were irrelevant. If the key genes (e.g. removed *Lacc15* from this network) were knocked-out, links of some genes (e.g. *Lacc2, Lacc4, Lacc16*) to this system will disappear. It means that these accessory genes might lose their roles to the function of lignin degradation. Whereas, removal of those 'random' genes (e.g. *Lacc1, Lacc5, Lacc6, Lacc7*) does not alter the main path structure of the remaining genes, and thus has no impact on the overall network topology. Therefore, in the engineering of increasing laccase activity, over-expressing these positive key genes (e.g. *Lacc3, Lacc13, Lacc14, Lacc15*), knock-out or down-regulating the negative key gene (e.g. *Lacc10*) is more effective than regulating the others, especially the genes irrelevant to the enzyme activity. Getting these key genes by statistical analysis and gene-gene interaction network could reduce blindness and save time in the process of finding functional genes and constructing engineering fungi.

The increased enzyme activity under Cu^{2+} stress, which was the systematic results of varied

genes and their interactions, was observed in this study as others found in *Pleurotus ostreatus* (32) and *Trametes pubescens* (16). These laccase genes were differentially expressed under Cu^{2+} stress. Although it is not completely understood how copper regulates laccase transcription in detail, in many cases, it is supposed to be associated with the putative metal-responsive elements (MREs) in the laccase promoter regions (31). In this study, we observed a similar interesting phenomenon as well that there was no MRE in the promoters of *Lacc8*, *Lacc14* and *Lacc16* (Fig. S2), which correlated to the laccase activity positively in the control group but showed no relation in the Cu^{2+} group. This may be another evidence to state that MRE is important for binding protein for laccase complex formation (33). No matter what the detailed regulation mechanism of Cu^{2+} is, the different expression suggested that in a practical application, the focused genes should be changed under different conditions. For example, under normal circumstances, we should aim to improve the expression of *Lacc8*, *Lacc13*, *Lacc14* and *Lacc15* to increase the laccase activity, while with the presence of Cu^{2+}, we should turn our attention to *Lacc3*，*Lacc10* and *Lacc13*.

For *Lacc1* and *Lacc6*, which showed no correlation with laccase activity in both group with relatively high transcript abundance (Fig. 4), may be involved in other physiological functions, such as fruiting body formation (34), stress response on diverse environmental challenges (35), or pathogenesis (36). In addition, due to the direct or indirect correlation with each other in both groups, *Lacc1* and *Lacc6* were suggested to play a role in synergy.

Conclusions

Network-based methods were applied to identify key functional genes and to outline

associations among genes and phenotype in a multi-gene family. This is an exploratory strategy to describe the transcriptional complexity of laccase and its relevant responses to Cu^{2+} stress. An interaction network in laccase multi-gene family was constructed to illustrate the relationship between two genes or gene-activity. The identified key functional genes associated with laccase activity (e.g. *Lacc10, Lacc13*) and the associations among genes and activity will help for the construction of high-yield laccase strains.

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Figure legends

Fig. 1 Time course of extracellular laccase activity detected in culture broth. The laccase activity was averaged from triple parallel measurements and expressed as units (U, 1 U corresponds to oxidize 1 μmol ABTS per minute). Error bars represent the standard deviation of the mean.

Fig. 2 PCA of the gene structures of the control and the Cu^{2+} group in different time courses. The number on each point is the value of the laccase activity that was detected on the day shown in the corresponding bracket.

Fig. 3 Correlation analysis of laccase enzyme activity and expression of gene *Lacc13* in the control and Cu^{2+} groups. An arrow points to the *Lacc13* concentration, which was determined on the sixth day in the Cu^{2+} group.

Fig. 4 The Kendall correlation network of the sixteen laccase genes and the total laccase activity (red) of the control (green) and Cu^{2+} group (blue). Only the correlations with absolute value no less than 0.6 are shown. Positive correlations are expressed in red and negative in black. The solid line means $p<0.05$, and the dotted line means $p>0.05$, whereas $|r| \geq 0.6$.

Figure 1

Time course of extracellular laccase activity detected in culture broth

The laccase activity was averaged from triple parallel measurements and expressed as units (U, 1 U corresponds to oxidize 1 μmol ABTS per minute). Error bars represent the standard deviation of the mean.

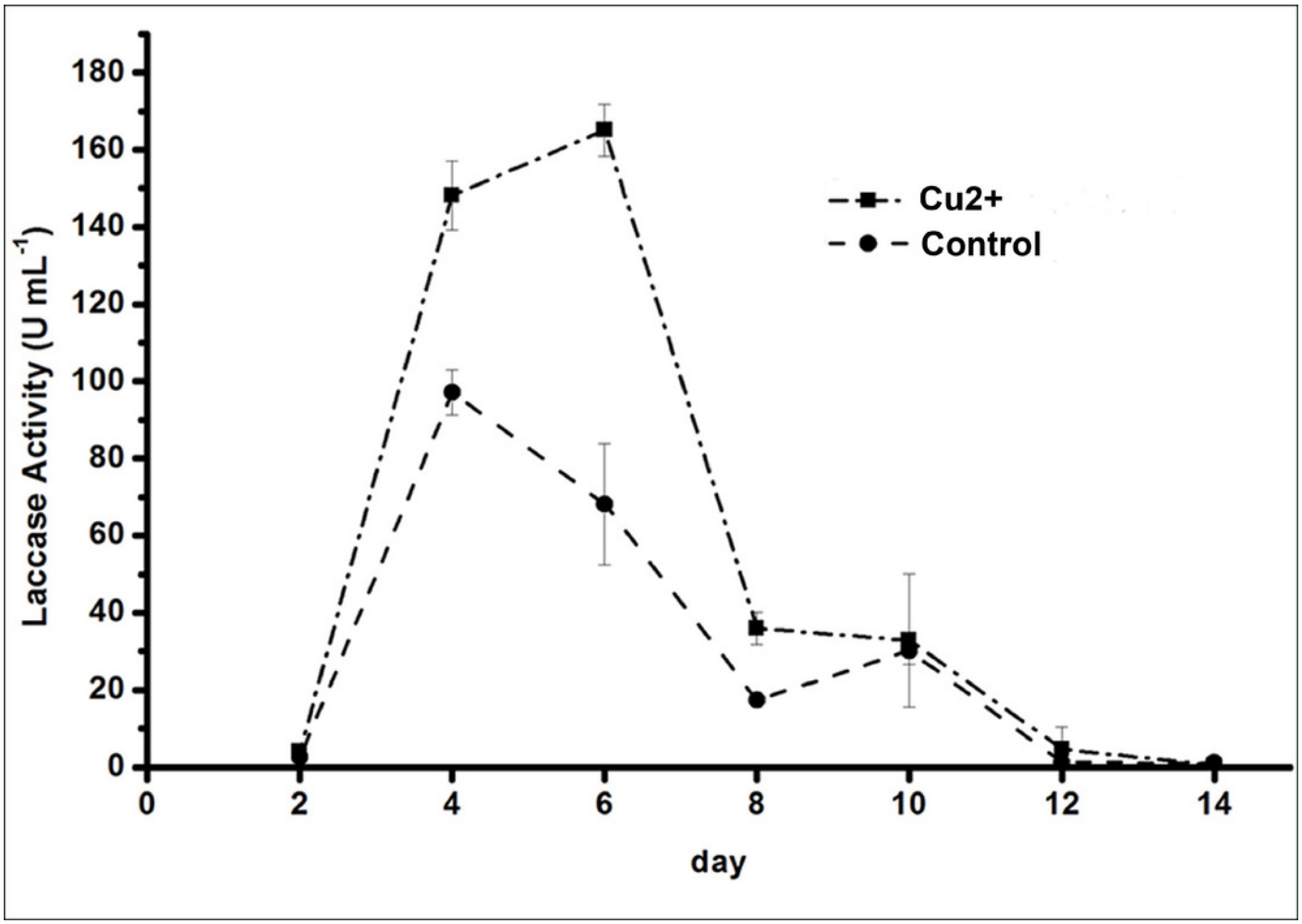

Figure 2(on next page)

PCA of the gene structures of the control and the Cu^{2+} group in different time courses

The number on each point is the value of the laccase activity that was detected on the day shown in the corresponding bracket

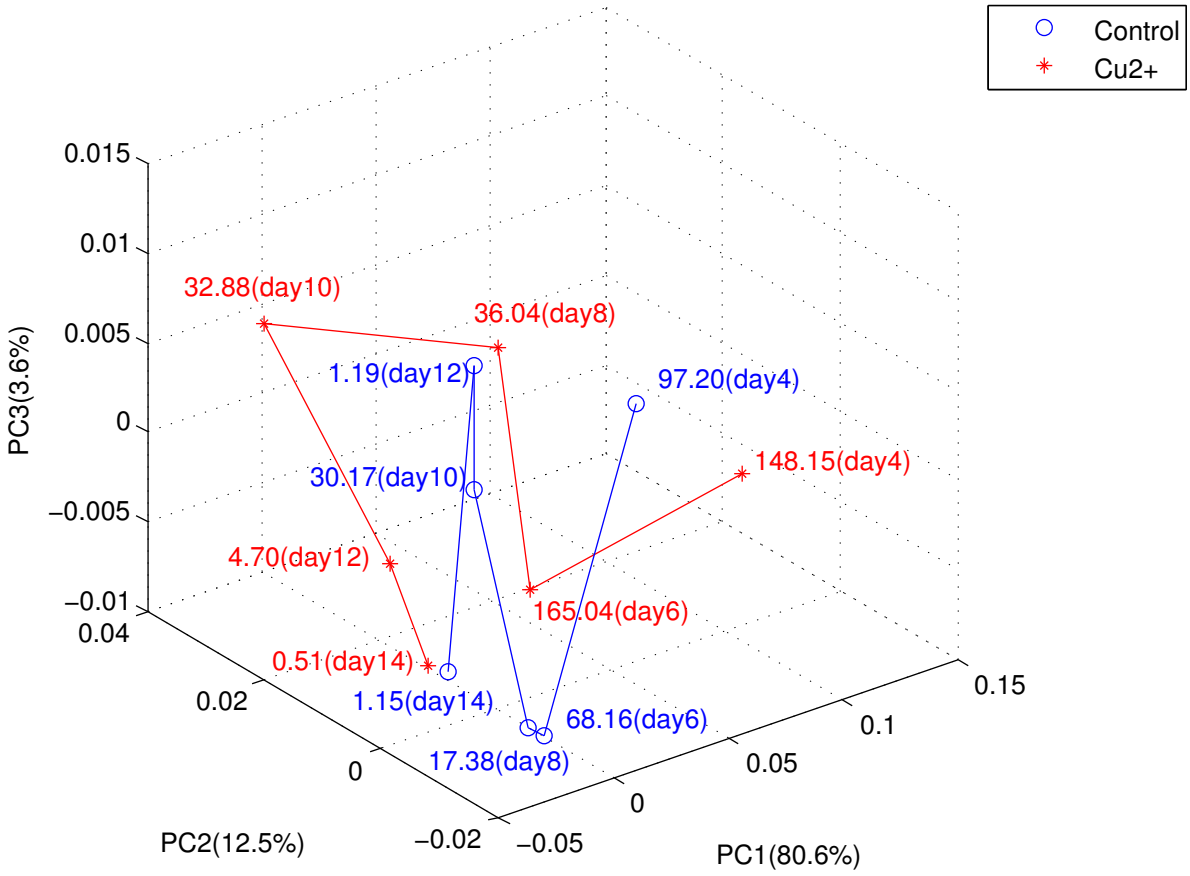

Figure 3(on next page)

Correlation analysis of laccase enzyme activity and expression of gene *Lacc13* in the control and Cu^{2+} groups

An arrow points to the *Lacc13* concentration, which was determined on the sixth day in the Cu^{2+} group

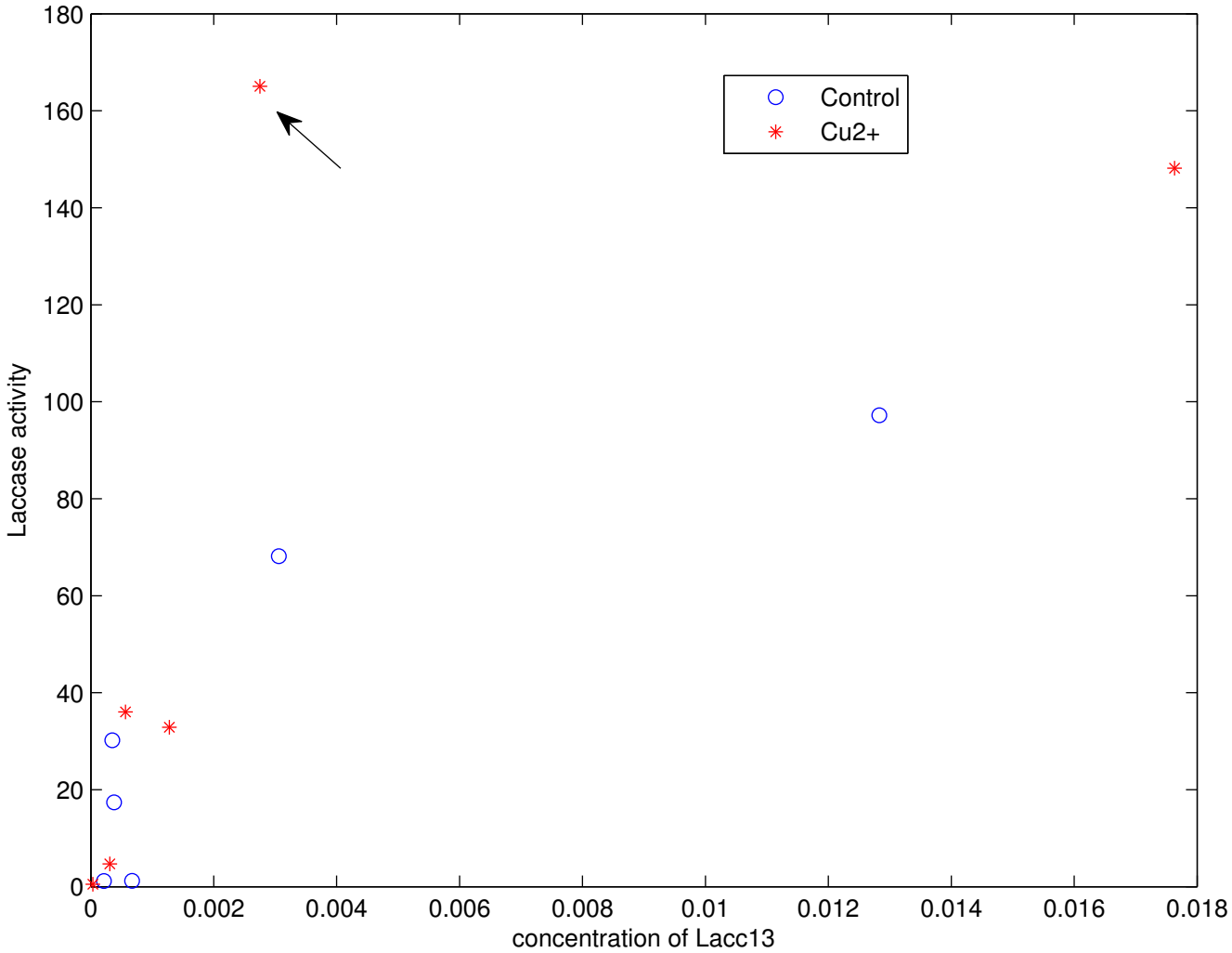

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Figure 4

The Kendall correlation network of the sixteen laccase genes and the total laccase activity (red) of the control (green) and Cu^{2+} group (blue)