## 2. MATERIALS AND METHODS

## 2.1 Media and culture conditions

**Oatmeal agar**

| | |
|---|---|
| Oatmeal agar | 36.5g |
| $H_2O$ | to 1L |

**YEG**

| | |
|---|---|
| Yeast extract | 2g |
| Glucose | 10g |
| $H_2O$ | to 1L |

**YEPD**

| | |
|---|---|
| Yeast extract | 5g |
| Peptone | 10g |
| Glucose | 10g |
| $H_2O$ | to 1L |

**Complete medium for *Magnaporthe grisea***

| | |
|---|---|
| Glucose | 10g |
| Peptone | 5g |
| Yeast extract | 1g |
| CAA | 1g |
| $NaNO_3$ | 0.6g |
| KCl | 0.5g |
| $MgSO_4$ | 0.5g |

| | |
|---|---|
| $KH_2PO_4$ | 1.5g |
| $H_2O$ | to 1L |
| pH | 6.5 |

**Minimal medium for *Magnaporthe grisea***

| | |
|---|---|
| Glucose | 10g |
| $NaNO_3$ | 0.6g |
| KCl | 0.5g |
| $MgSO_4$ | 0.5g |
| $KH_2PO_4$ | 1.5g |
| $H_2O$ | to 1L |
| pH | 6.5 |

**AB minimal medium for *Agrobacterium***

| | |
|---|---|
| AB liquid | 90ml |
| 20 x AB buffer | 5ml (1 x) |
| 20 x AB salts | 5ml (1 x) |

| **AB buffer** | **(20 x)** |
|---|---|
| $K_2HPO_4$ (anh.) | 6g |
| $NaH_2PO_4$ (anh.) | 2g |
| $H_2O$ | to 100ml |

Each salt was dissolved separately in ~ 50ml $H_2O$ and then mixed to obtain 100ml solution with pH 7.0.

| **AB salts** | **(20 x)** |
|---|---|
| $NH_4Cl$ | 2g |
| $MgSO_4.7H_2O$ | 0.6g |
| KCl | 0.3g |
| $CaCl_2$ (anh.) | 0.3g |
| $FeSO_4$ | 0.005g |
| $H_2O$ | to 100ml |

**AB liquid**

0.5 g glucose in 90ml $H_2O$

**Induction medium for *Agrobacterium***

| | |
|---|---|
| 20 x AB salts | 5ml (1 x) |
| Glucose | 180mg (10mM) |
| Glycerol | 0.5 % w/v |
| $H_2O$ | to 100ml |
| pH | 5.3 |

**Synthetic Dropout (SD) medium for yeast**

| | |
|---|---|
| YNB without amino acids (Hi-Media, India) | 1x |
| Ethanol | 2% |
| $H_2O$ | to 1L |

## 2.2 Bacterial strains

*Escherichia coli* DH5α ($F^-$, *end*A1, *hsd*R17 ($r_k^-$, $m_k^+$), *sup*E44, *thi*-1, $\lambda^-$, *rec*A1, *gyr*A96, relA1 Δ(*lacZYA-arg*F) U169 *deo*R (ϕ80d*lac*Δ(lacz) M15; Bethesda Research Laboratories) was used for bacterial transformation and plasmid propagation. The *E. coli* strains were grown at $37^0$C on Luria-Bertani (LB) agar medium. *E. coli* transformants carrying plasmid vectors were grown on LB agar containing either Ampicillin or Kanamycin at a concentration of 100μg/ml or 50μg/ml, respectively, as required. Bacterial strains were maintained at $4^o$C as slants or stab cultures on LB agar medium. Long-term preservation was under 25% glycerol solution at $-70^o$C.

## 2.3 Yeast strains

*Saccharomyces cerevisiae* strain s288C (*MAT* α, *SUC2*, *gal2*, *mal*, *mel*, *flo1*, *flo8-1*, *hap1*, *ura* 3-52) was used for yeast transformation. The yeast strain was grown at 28-$30^0$C and maintained on YEPD medium. Ampicillin to a final concentration of 200μg/ml was added to the autoclaved medium to avoid bacterial contamination.

## 2.4 Fungal cultures

*Magnaporthe grisea* B157, belonging to the international race IC9 was isolated in our laboratory from infected rice leaves (Kachroo *et al*., 1994). The fungus was grown on either YEG agar or Oat meal agar, at 28-$30^0$C. Fungal conidia were harvested by scraping the biomass grown on Oatmeal agar plates with a sterile surgical blade, resuspended in sterile water, and purified by passing through glass wool column. The

different transformants were maintained as monoconidial isolates and stored as 25% spore glycerol stocks at -70$^0$C.

## 2.5 Competent cell preparation and transformation of *E. coli* with plasmid DNA

Single colony of *E. coli* DH5α was inoculated in 3ml of Luria-Bertani broth and grown overnight. 100ml of fresh LB broth was inoculated with 1ml of overnight grown culture and grown at 37$^0$C till 0.3 to 0.4 O.D$_{600}$; chilled on ice and the cells were pelleted by centrifugation at 2012X g for 5min at 4$^0$C. The cells were resuspended in ice-cold 0.1 M $CaCl_2$ and incubated for 30min. The cells were reharvested by centrifugation and resuspended in ice-cold 0.1M $CaCl_2$ with 20% glycerol. The culture was stored at -70$^0$C in 100µl aliquots. For transformation, a 0.2ml aliquot of this cell suspension was transferred to a 1.5ml chilled microfuge tube; mixed with the plasmid DNA (usually 50ng) or ligation mixture and incubated at 4$^0$C for 30min. Cells were subjected to heat shock at 42$^0$C for 90sec, followed by 5min. incubation on ice. 1ml of LB broth was added to the above suspension and incubated at 37$^0$C for 30min. About 0.1ml aliquot of transformation mixture was plated on LB agar medium containing either ampicillin (100µg/ml) or kanamycin (50µg/ml).

## 2.6 Transformation of *S. cerevisiae* with plasmid DNA

Yeast strain, s288C, was transformed with the plasmid by 'One-step transformation' method (Chen *et al*., 1992). The yeast culture was spread on a YEPD plate and incubated for 20-24h. A small part of the growing culture was transferred using a sterile toothpick

to a microfuge tube containing 100µl of 45% PEG 4000 (solution in 0.1M Lithium actetate, ph. 6.0, 0.1M DTT), 5µl of 10µg/ml of calf thymus DNA as carrier, which was sheared to give molecules in the 500bp range. 1µg of transforming DNA was added to each tube. The whole mixture was thoroughly vortexed and was given heat shock at $39^0$C for 1h. The cells were then gently spread on predried selection medium plates.

## 2.7 Isolation of plasmid DNA

Overnight grown culture (1.5ml) was centrifuged in a microfuge tube and the bacterial pellet was resuspended in 200µl of STET (8% Sucrose, 5% TritonX-100, 50mM Tris pH 8.0, 50mM EDTA pH 8.0). Lysozyme (25µl of 10mg/ml) was added to it, mixed by vortexing and boiled in a water bath for 45sec followed by centrifugation at 13000X g for 10min. The resulting snot was removed with a tooth pick and 20µl of 5% CTAB was added followed by centrifugation for 5min. To the pellet, 300µl of 1.2M NaCl was and 750µl of 100% ethanol was added. The DNA pellet was obtained by centrifugation for 10 min; washed with 70% ethanol and resuspended in 50µl of TE (10mM Tris pH 8.0, 1mM EDTA pH 8.0).

## 2.8 Nucleic acid manipulations

Restriction enzyme digestion of DNA was carried out in reaction buffers, supplied with the corresponding enzymes, as recommended by the suppliers. Blunt ended ligations were carried out at $20^0$C and sticky end fragments were ligated at $16^0$C for 3-4 h.

## 2.9 Heterologous expression of *Mg*Lac1 and *Mg*Lac2 in *Saccharomyces cerevisiae*

*MgLac1* and *MgLac2* genes were PCR amplified (Forward primer: 5'ATGAATCTTCGGGACACCATCT 3'; Reverse primer 5' TTATCTCCTCAAACCAGACTCCA 3' and Forward primer: 5'GGGGATCCCGATGGGTATCATGCAGGGGATG3'; Reverse primer 5'GCAAGCTTGGTTAAACACCGCTGTCGATCTG3' respectively) and cloned into yeast expression vector 'pEG (KT)' (Mitchell *et al*., 1993) so that they were translationally 'in frame' with Glutathione-S-Transferase (*GST*) gene. Yeast strain, s288C, was transformed with the recombinant plasmid by 'one-step transformation' method (Chen *et al*., 1992). Four percent galactose was used to induce expression of GST*Mg*Lac1 and GST*Mg*Lac2 fusion proteins in the yeast transformants.

## 2.10 Enzyme purification

The GST*Mg*Lac1 and GST*Mg*Lac2 fusion proteins were purified from total protein extract of the transformed *S. cerevisiae* following induction by galactose (4%, w/v). The GST fusion proteins were easily purified by affinity chromatography using a glutathione-sepharose matrix under mild conditions, using the GST purification module as per manufacturer's instructions (GE Healthcare, Buckinghamshire, UK).

## 2.11 Thrombin protease cleavage

Removal of the GST moiety from the proteins of interest was accomplished through a thrombin protease cleavage site located between the GST moiety and the recombinant proteins. GST from the fusion protein was removed using Thrombin Cleavage Capture

Kit (Novagen, Merck KGaA, Darmstadt, Germany) as per the manufacturer's instructions. Cleaved GST was easily removed by a second round of chromatography on the glutathione column. The purified proteins were checked on 12% SDS–PAGE.

## 2.12 Raising antibodies against *Mo*Lac1 and *Mo*Lac2

The purified protein was used to raise polyclonal antibodies in rabbit. 5µg of purified *Mg*Lac1 and *Mg*Lac2 in 0.5ml of 1X PBS was emulsified with an equal volume of Freund's complete adjuvant and was used immediately for subcutaneous injection in rabbit. Two booster doses of antigen- adjuvant mixture were given after every 4 weeks. 15ml of blood were drawn 7days after the second booster dose. The antibody titer was estimated by indirect-enzyme linked immuno sorbent analysis (ELISA) using anti-rabbit IgG (Bangalore Genie, India) as the secondary antibody.

## 2.13 Protein extraction and western blotting

Total intracellular protein was extracted from wild type *M. grisea* mycelium grown in CM for 24h. *M. grisea* biomass was crushed into fine powder in liquid nitrogen and resuspended in 1X PBS containing 1mM PMSF. The extract was clarified by centrifuging at 13414X g for 15min at $4^0$C. For extracellular proteins culture filtrate was concentrated with an Amicon Stirred Cell protein concentrator with a 3kDa cutoff. Protein concentration was estimated by Bradford method (Bradford, 1976). The protein samples were electrophoresed on 10% SDS- polyacrylamide gels, followed by electrotransfer to nitrocellulose membrane (Hybond ECL, GE Healthcare, Buckinghamshire, England). The membrane was incubated in the blocking solution (3% BSA, 0.02% $NaN_3$, 0.2%

Tween 20 and 1X PBS) for 2h. The blocking solution was discarded and the membrane was incubated with primary antibody (Rabbit anti-*Mo*Lac1and anti-*Mo*Lac1 antibody) diluted to 1:500 in blocking solution, for 2h and then washed 5 times (20min each) with the blocking solution. Subsequently, the membrane was incubated with the alkaline phosphatase labeled secondary antibody (Goat anti-rabbit IgG antibody; Bangalore Genei, Bangalore) diluted to 1:1000 in secondary solution (0.15M NaCl; 0.05M Tris; 0.2% Tween 20, 0.02% $NaNO_3$ and 5% skim milk powder), for 1h and the blot was then washed 4-5 times (20min each) in secondary solution. The immunoblots were developed by adding alkaline phosphatase buffer (0.5M NaCl; 5mM $MgCl_2$; 0.1M Tris, pH 9.5) containing 66µl of (10mg/ml) nitroblue tetrazolium (NBT) and 33µl of (10mg/ml) 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

## 2.14 Enzyme assays

### 2.14.1 ABTS assay method for laccase

This assay method is commonly used and is described by Bourbonnais and Paice (1990). The nonphenolic dye 2,2'-azinobis-bis-(3-ethylbenzthiazolinesulphonate) (ABTS) is oxidized by laccase to the more stable and preferred state of the cation radical (**Figure 7**). The concentration of the cation radical responsible for the intense blue-green colour can be correlated to enzyme activity (Macherczyk *et al.*, 1995) and is most often read between 415nm and 420nm.

ABTS (0.4mM) was dissolved in sodium acetate buffer (pH 4.5; $25^0$C). The absorbance of the cation radical was monitored at 420nm (emM = 36 /mM /cm) and $25^0$C (Palmieri

*et al.*, 1997) using a Unicam UV/Vis spectrophotometer. Enzyme activity was expressed as international units (IU) where 1 IU is defined as the amount of enzyme forming 1 mole of product per minute. The reaction mixture contained 580ml of substrate and 20ml of enzyme or sample.

### 2.14.2 Syringaldazine assay method for laccase

This assay method is adapted from a method described by Harkin and Obst (1973). It is based on the oxidation of 4,4'-[azinobis(methanylylidene)]bis(2,6- dimethoxyphenol) (syringaldazine) to the corresponding quinone, 4,4'- [azinobis(methanylylidene)]bis(2,6-dimethoxycyclohexa-2,5-diene-1-one) (**Figure 8**). An increase in absorbance at 530nm is followed at $25^0$C to determine laccase activity in international units (IU) where 1 IU is defined as the amount of enzyme forming 1 mole of product per minute.

A stock solution of Syringaldazine was prepared by dissolving the substrate over a period of 3 hours in 96 % ethanol. Syringaldazine for analysis was prepared by adding deionised water to the stock solution until the desired concentration of 0.28mM was achieved (Felby., 1998). The reaction mixture consisted of 1 ml buffer (Sodium phosphate at the desired pH), 0.075ml substrate and 0.025ml enzyme.

## 2.15 Substrate specificity

The specificity of laccase toward different phenolic compounds was investigated. Substrates were chosen according to the positions of substituents on the phenolic ring and the type and/or length of the substituents. Three of the most commonly used substrates

(ABTS, DMP and Syringaldazine) (Palmieri *et al*., 1997; Giardina *et al*., 1999; Xu., 1996) for laccase determination were evaluated. Seven other phenolic compounds (Guaiacol, Pyrocatechol, Hydroquinone, Pyrogallol, Phloroglucinol, Ferulic acid and Tyrosine) were evaluated. All the substrates were purchased from Sigma Chemical, St. Louis, MO, USA.

The substrates were dissolved in Sodium Acetate buffer, pH 5, at concentrations of 1mM to 10mM. The reaction mixtures contained 50ml of enzyme that was added to 950ml of substrate to start the reaction. The oxidation of the substrates was followed during a wavelength scan on the Unicam UV/Vis spectrophotometer in the visible region (300nm to 700nm). The wavelength where maximal absorbance increase over time was observed was recorded and used to measure the oxidation rate during kinetic studies.

Kinetic studies were conducted for each substrate that could be oxidized by the laccase enzymes. At least eight different substrate concentrations for each substrate were assayed for each laccase at the optimal pH for each enzyme. Triplicates of each assay were done. The data was subjected to nonlinear regression analysis (Graph Pad Prism Software, La Jolla, CA, USA) using the Michaelis-Menten equation and the kinetic parameters, Km and Vmax were determined. The wavelengths of maximum absorbance for the oxidised substrates by the isolated laccases are reported in **Table 1.**

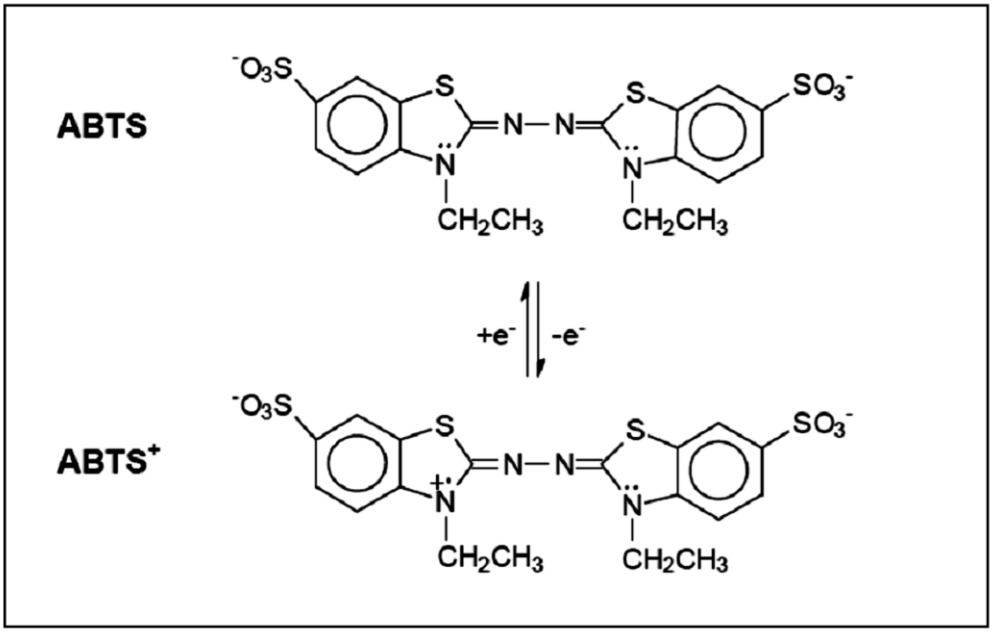


**Figure 7:** The laccase-catalysed oxidation of ABTS to a cation radical (ABTS+) (Macherczyk *et al.*, 1998).

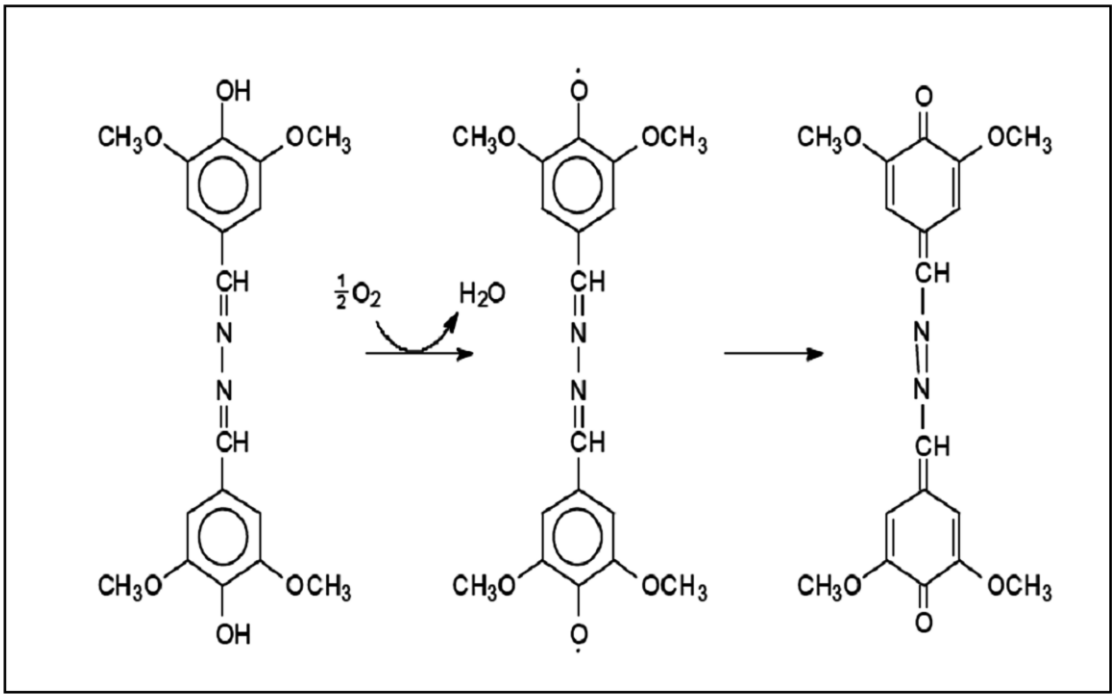


**Figure 8:** The laccase-catalysed oxidation of Syringaldazine to its corresponding quinone (Sanchez-Amat and Solano., 1997).

## 2.16 Inhibition studies

Four potential inhibitors were evaluated to test the inhibition properties of laccases. These inhibitors included: sodium azide ($NaN_3$), cysteine, EDTA and halide ($Cl^-$). The standard test of inhibitory action was performed by preincubation of the enzyme with the inhibitor substance for a certain time (2-10min) to ensure that the inhibition is complete. The subsequent kinetic measurement was started by the addition of proper substrate and then their decline or the formation of the product was measured. Triplicates of each assay were done. The data was subjected to nonlinear regression analysis (Graph Pad Prism Software, La Jolla, CA, USA) using the Dose-response - Inhibition equation and the kinetic parameter (IC50) was determined.

## 2.17 pH optimum

The influence of pH on laccase activity was studied spectrophotometrically. Three different substrates were used to determine the optimum pH of laccases, because it is known that laccases have different optimum pH values for different substrates (Xu., 1996).The three substrates used were: ABTS, Catechol and Pyrogallol. The pH optima were determined over a range of pH 3 to 9. A 0.1 M Britton - Robinson buffer (Xu, 1996) was used for the entire pH range. The Britton-Robinson buffer was made by mixing 0.1 M boric acid, 0.1M acetic acid and 0.1M phosphoric acid and adjusting the pH with 0.5M NaOH. The different assays for the different substrates were conducted as described under assay methods. All assays were done in triplicate.

| Substrates | λ (max. change, nm) |
|---|---|
| ABTS | 420 |
| 2,6-DMP | 477 |
| Syringaldazine | 530 |
| Guaiacol | 468 |
| Catechol | 390 |
| Pyrogallol | 390 |
| Hydroquinone | 390 |
| Tyrosine | 280 |
| Ferulic acid | 287 |
| Phloroglucinol | 320 |

**Table 1:** Wavelengths of maximal absorption of laccase-oxidisable aromatic compounds.

## 2.18 Temperature effect and thermostability

The effect of temperature on laccase activity was determined by spectrophotometrically following the laccase-catalysed oxidation of ABTS for 2 min at temperatures ranging $25^0$C to $100^0$C at $5^0$C intervals. Britton Robinson buffer (0.M) was used for all the reactions, at the optimum pH for Catechol. The substrate was incubated for at least 2 min at the different temperatures before the enzyme was added to start the reaction. The thermal stability of the laccase was determined by following the oxidation of ABTS at $25^0$C after pre incubation of laccase for 1hour at different temperatures ($20^0$C to $70^0$C). The reaction was started by the addition of substrate.

## 2.19 Dye decolourisation assay

Dye decolourising activity was estimated by diluting 20ml of a 0.2% (wt/vol) Remazol brilliant blue R (Sigma Chemical, St. Louis, MO, USA) dye solution (in $H_2O$) to 4ml with pH 4.5 citrate-phosphate buffer. Laccase (400U) was added, and decolorisation was assayed after 0, 10, 20, 30, 45, 60, 75 and 90min at $23^0$C. Decolorisation was monitored by diluting 0.5ml of the dye solutions to 2ml with a 10mM sodium azide solution in 10mM pH 4.5 citrate-phosphate buffer. The ratio of absorbance ($A_{591}/A_{500}$) was measured for the azide-dye solution (Lozovaya *et al*., 2006**)**. Decolorization assays were performed in triplicate, and absorbances were compared with that of a control without added laccase (Edens *et al*., 1999).

## 2.20 DHN polymerisation potential

Polymerization of Chromotropic acid disodium salt dehydrate (1,8- Dihroxynapthalene-3,6-disulfonic acid disodium salt; 4,5- Dihroxynapthalene-2,7-disulfonic acid disodium salt) (Sigma Chemical, St. Louis, MO, USA), was measured by the addition of 15U of laccase to a 2ml solution of 1mM DHN in 5mM pH 4.5 citrate-phosphate buffer in 50% ethanol. The polymerization solution was incubated at $23^0$C and monitored spectrophotometrically by scanning the solution from 320 to 520nm at 0 to 120min. An identical control solution without the enzyme that auto-oxidized was also scanned (Edens *et al*., 1999).

## 2.21 Iron oxidase activity of recombinant laccase

Ferroxidase activity was determined using ferrous sulfate as the electron donor and 3-(2-pyridyl)- 5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine) as a specific chelator to bind ferrous iron remaining at the end of the reaction. Reactions were carried out in disposable cuvettes containing 100μM ferrous sulfate in 100mM sodium acetate buffer (pH 5.0). The reactions were quenched by addition of ferrozine to a final concentration of 1.5mM, and the rate of $Fe^{2+}$ oxidation was calculated from the decreased absorbance at 560 nm using a molar absorptivity of $e_{560}$= 25,400 $M^{-1}$ $cm^{-1}$ for the $Fe^{2+}$–ferrozine complex (Hoopes and Dean., 2003).

## 2.22 Construction of *MgLac1* antisense vector

The *MoLac1* was amplified by PCR using the forward primer: 5’ATGAATCTTCGGGACACCATCT 3’ and reverse primer 5’

TTATCTCCTCAAACCAGACTCCA 3'. Thermal cycling conditions consisted of 5min at $94^0$C followed by 30 cycles of 30sec at $94^0$C, 30sec at $60^0$C, 3min at $72^0$C and a final extension of 5min at $72^0$C in a thermal cycler (Eppendorf, Hamburg, Germany). The PCR amplified product was gel purified and was digested with *Xho*I and *Hind*III and cloned into pSILENT vector (Nakayashiki *et al*., 2005) digested with the same enzymes, creating plasmid pSILENT*MoLac1*. A 1508bp *MoLac1* region from 560bp to 2067bp was used for the antisense construct. Plasmid pSILENT*MoLac1* was then digested and cloned at *Xba*I site in binary vector pCAMBIA-1305.2 to obtain the final construct.

## 2.23 Construction of *MgLac2* antisense vector

The *MoLac2* was amplified by PCR using the forward primer: 5'GGGGATCCCGATGGGTATCATGCAGGGGATG3' and reverse primer 5'GCAAGCTTGGTTAAACACCGCTGTCGATCTG3'. Thermal cycling conditions consisted of 5 min at $94^0$C followed by 10 cycles of 1min at $94^0$C, 45sec at $65^0$C, 2min at $72^0$C and 25 cycles of 1min at $94^0$C, 30sec at $50^0$C, 2 min at $72^0$C a final extension of 5min at $72^0$C in a thermal cycler (Eppendorf, Hamburg, Germany). The PCR amplified product was gel purified and was digested with *Bgl*II and *Kpn*I and cloned into pSILENT vector (Nakayashiki *et al*., 2005) digested with the same enzymes, creating plasmid pSILENT*MoLac2.* An 819 bp *Mo*Lac2 region from 506 bp to1324 bp was used for the antisense construct. Plasmid pSILENT*MoLac2* was then digested and cloned at *Xba*I site in binary vector pCAMBIA-1305.2 to obtain the final construct.

## 2.24 Agrobacterium tumefaciens mediated transformation of M. grisea

The *Agrobacterium* strain LBA4404 / pSB1 (Komari *et al*., 1996) was first transformed with respective vectors *via* triparental mating (Helper plasmid pRK2013; Helper strain E. coli DH5α). For tri parental mating, single colonies, each from the freshly grown recipient *Agrobacterium* LBA4404/pSB1, helper plasmid pRK2013 and the donor plasmid were mixed together on YEP agar (Yeast extract 0.2%, Peptone 0.5%, agar 1.5%) plates and incubated at $28^0$C for 18-20h. The mixture was scraped and resuspended in 0.9M NaCl. 100µl of serial dilutions of this suspension were plated onto selection plates and incubated at $28^0$C for 2-3 days till the appearance of single colonies. The *Agrobacterium* harbouring T-DNA vector was then used to transform the fungus. *Agrobacterium tumefaciens* mediated transformation (ATMT) of *M. grisea* was carried out as described (Mullins *et al*., 2001). Briefly, *M. grisea* spores were collected from 7-8 days old oatmeal agar plates and spore concentration was adjusted to $1x10^6$ spores/ml. *Agrobacterium* carrying the *hpt* gene expression cassette was grown at $28^0$C for 2 days in AB minimal medium supplemented with appropriate antibiotics. The *Agrobacterium tumefaciens* cells were diluted to $OD_{600}$ = 0.15 in induction medium (IM) and grown for another 6h, both in the presence (IM+AS) and absence (IM-AS) of 200µM acetosyringone (AS). The cells were grown for an additional 6h before mixing them with an equal volume of a conidial suspension from *M. grisea* ($1 \times 10^5$ conidia/ml). This mix (200µl/plate) was plated on a 0.45µm pore, 45mm diameter nitrocellulose filter (Millipore, Bangalore, India) placed on co-cultivation medium (same as IM except that it contains 5mM glucose instead of 10mM glucose) in the presence of 200µM AS.

Following incubation at $28^0$C for 2 days, the filter was transferred to YEG containing Hygromycin B (Sigma Chemical, St. Louis, MO, USA) to the final concentration of 200 µg/ml. as a selection agent for transformants and cefotaxime (250µg/ml) to kill the *Agrobacterium tumefaciens* cells. Wild type *M. grisea* was kept as a control which did not grow on hygromycin containing medium. The transformants were maintained as monoconidial isolates to get pure cultures. Spores from these monoconidial cultures were stored in 25% glycerol at $-70^0$C until further analysis.

## 2.25 DNA extraction and Southern blot analysis

Total DNA from *M. grisea* was extracted as described by Dellaporta *et al*., (1983). Approximately 5g biomass was taken from culture grown in CM for 3-4 days, frozen in liquid nitrogen and ground into a fine powder in a pre chilled mortar and pestle. The powder was transferred to a 50ml centrifuge tube containing 15 ml of extraction buffer (0.1M Tris, pH 8.0; 0.05M EDTA, pH 8.0; 0.5M NaCl; 0.01M β-mercaptoethanol). To this mixture, 1ml of 20% SDS was added, mixed and the tubes were incubated at $65^0$C for 10min. Thereafter, 5ml of 5M potassium acetate was added, the tubes were shaken gently and were incubated on ice for 20min followed by centrifugation at 2012X g for 30min. The supernatant was collected in a clean 50ml centrifuge tube containing 10ml of isopropanol, mixed and the tubes were incubated at $-20^0$C for 30min. The DNA was pelleted down at 2000X g for 30min and the supernatant was gently poured off. Remaining liquid was removed by keeping the tubes inverted and the pellets were then air dried. The DNA was redissolved in 700µl of sterile distilled water, transferred to 1.5

ml microfuge tube and centrifuged to remove all the insoluble debris. 5 µl of RNase (10mg/ml) was added to the DNA solution and was incubated at $68^0$C for 10min. Further, 400µl of chloroform: isoamylalcohol (24:1) was added to these tubes, mixed well and spun for 1min in microcentrifuge. The top aqueous phase was transferred to another 1.5ml tube. To this solution, 75µl of 3M sodium acetate, pH 5.2, and 500µl of isopropanol was added, mixed well, and DNA was pelleted by centrifugation in a microfuge. The pellet was washed with 70% ethanol, dried and redissolved in 100µl of sterile distilled water.

Southern blot was prepared as described by Sambrook *et al.*, (1989). The genomic DNA (10 µg) from wild type and different transformants was digested with restriction enzymes as recommend by the supplier; electrophoresed on 0.8% agarose gels and blotted onto nylon membrane (Hybond $N^+$, Amersham Biosciences, Buckinghamshire, England). DNA was fixed to the membranes by UV crosslinking ($12x10^4$ µJ/$cm^2$) using UV cross-linker (Spectrolinker, Spectronics Corporation, USA).

Hybridisation probe was labeled nonradioactively using Gene Images AlkPhos Direct Labelling and Detection System as per manufacturer's instructions (Amersham Biosciences, Buckinghamshire, England). Filters were exposed to X-ray films with intensifying screen for appropriate time at $-70^0$C. Standard procedures were followed for autoradiography.

## 2.26 Total RNA extraction and RT-PCR (Reverse Transcriptase Polymerase Chain Reaction)

Fungal biomass grown in liquid media was frozen in liquid nitrogen. Total RNA was isolated using TRIZOL reagent (Invitrogen Life Technologies, California, USA). The quality of isolated RNA was checked by electrophoresis on formaldehyde gels and quantified by UV spectrophotometry. 5µg of total RNA was used to synthesise the first strand cDNA using MuMLV reverse transcriptase (Fermentas GmBH, St. Leon-Rot, Germany) and random hexamer in 20µl reaction system.

## 2.27 Quantitative real-time PCR (qRT-PCR)

For Quantitative real-time PCR (qRT-PCR), total RNA was extracted from the samples, treated with RNAase-free DNase, and 1µg was reverse-transcribed, in the presence of random hexamer primer. qRT-PCR was performed by monitoring the increase in fluorescence of the SYBR Green dye on LightCycler system (Roche Applied Science, Mannheim, Germany), according to the manufacturer's instructions. Each qRT-PCR quantification was carried out in triplicate using primers for each individual gene (**Table 2**). The primer efficiencies were ~95-100%. Thermal cycling conditions consisted of 2min at $95^0$C followed by 40 cycles of 10sec at $95^0$C, 10sec at $54^0$C, and 10sec at $72^0$C. The data were normalized against *tubulin* gene, and fold change in the expression level was calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen., 2001).

The fold induction was calculated by the formula:

$\Delta\Delta Ct = \Delta Ct$ (Transformed sample) – $\Delta Ct$ (Wild type sample)

ΔΔCt: Fold induction

Ct: Threshold cycle or the cycle number when change in fluorescence is detected

ΔCt (Transformed sample): Ct, target gene – Ct, *tubulin*

ΔCt (Wild type sample): Ct, target gene – Ct, *tubulin*

Fold induction = $2^{-\Delta\Delta Ct}$

## 2.28 Phenotypic characterisation of the knockdown transformants

### 2.28.1 Growth and conidiation

The transformants and wild type strain were grown on OMA (oatmeal agar) and growth diameter was measured after 4 days, 6 days and 8 days. Dry weight was also estimated after growth in CM for 7 days. Conidia were collected from 7-8 days old OMA plates with 1ml of distilled water and counted using a haemocytometer.

### 2.28.2 Appressorial assay

Appressorial assays were carried out *in vitro* using hydrophobic side of the gel bond film (Lee and Dean., 1994). Approximately 50µl of $10^4$ spores/ml were placed on hydrophobic side of gel bond film (GE Healthcare, Buckinghamshire, England) and incubated under moist conditions for 12-16h. The spores were checked for appressorium formation under microscope.

| Gene id | Primer sequence | |
|---|---|---|
| MGG_08127.5 | Forward | 5' CCTGCCAGCGCGAATTACG 3' |
| | Reverse | 5' CGACCTCCACTGCCTTTGGG 3' |
| MGG_02876.5 | Forward | 5' AAGACGGTGTGCCTGGTGTGA 3' |
| | Reverse | 5' AGAAGACCATTGGGCCAACG 3' |
| MGG_13464.5 | Forward | 5' ACCACTCTCACTTCTCCGGG 3' |
| | Reverse | 5' GGATCAGCTCGTACTGGATGCG 3' |
| MGG_0579.5 | Forward | 5' CGGGCTCGACCGTGACTTA 3' |
| | Reverse | 5' TTCCCAGGTCCTCGTCGTAGTT 3' |
| MGG_09139.5 | Forward | 5' ATGTATGGCGGCATCGTCATCA 3' |
| | Reverse | 5' GGCCGTTGGACAGAATCGGAG 3' |
| MGG_11608.5 | Forward | 5' ACGTGACCAACAACATGCAGAC 3' |
| | Reverse | 5' GGGTGGCGCGGAACTTGTA 3' |
| MGG_00551.5 | Forward | 5' CAGATGGTGTTGTCCGCGAT 3' |
| | Reverse | 5' TTCTGCTGAATACCGTGCCAGTG 3' |
| MGG_07771.5 | Forward | 5' AGCGGGACAGCGTTCAAAT 3' |
| | Reverse | 5' ATAGGGCCGTACAACCCATCG 3' |
| MGG_07220.5 | Forward | 5' CGGCTCCAACGAGATGGAT 3' |
| | Reverse | 5' CGATGATAAGAGGACCACGCAG 3' |
| MGG_02156.5 | Forward | 5' GTCCCATGAAGGAGCTGATCGC 3' |
| | Reverse | 5' ACGTTGACCATCCTGACCAGGTA 3' |
| MGG_14307.5 | Forward | 5' ACTATGAGATCCGACCCGACATT 3' |
| | Reverse | 5' CCAGGCCAACAACAACGTCC 3' |
| MGG_09102.5 | Forward | 5' GCATCGACGAGCACGAGTTC 3' |
| | Reverse | 5' GTCACCCTCACGGCGTAGT 3' |
| β Tubulin | TubL1 | 5' GAGTCCAACATGAACGATCT 3' |
| | TubR1 | 5' GTACTCCTCTTCCTCCTCGT 3' |

**Table 2:** Primers used for the quantitative real-time RT-PCR

### 2.28.3 Melanin estimation

Total melanin was estimated as melanin has been implicated as a pathogenicity factor in *M. grisea* (Woloshuck *et al*.,1980). Fungus was grown on yeast extract medium for 7-10 days. Melanin was quantitated by crushing biomass in liquid nitrogen and solubilising it in 1ml of 1N NaOH for 1h at $80^0$C and then centrifuged at 12,000X g for 10min. The absorbance of the supernatant was measured at 405nm. Melanin content was compared and expressed as a percentage of control value.

## 2.29 Infection assays

Barley explant infection was carried out was described (Clergeot *et al*., 2001), where 3-4 week old leaves were taken for infection assay. The leaf segments were placed on water agar plus kinetin (2mg/L) plates, inner side of the leaf upwards. Using cotton buds, the surface of the leaves was thoroughly humidified with water by brushing over the leaf in one direction. ~30 µl droplets of fungal spore suspension (~$5x10^4$spores/ml) were put on leaf segments and disease symptoms were recorded after 4-5 days. The invasive growth was examined by abrading the leaves with a needle before inoculating the spore suspension.

For spray inoculation of barley, conidial suspensions were adjusted to $5\times10^4$ spores/ml and spray inoculated on barley of three- to four-leaf stage. Inoculated plants were kept in humidity chamber and blast disease development was monitored. Disease symptoms were recorded 4-5 days after inoculation.

## 2.30 Penetration assay

The onion penetration assay was performed as previously described (Xu *et al*., 1998). Onion epidermal strips were isolated and 15µl of conidial suspension ($5\times10^4$ spores/ml) was inoculated onto the adaxial surface. After 48h of incubation, onion epidermal strips were fixed with lactophenol (lactic acid/phenol/glycerol/water = 1:1:1:1) and infection hyphae and host cell were observed under bright field microscope.

## 2.31 Sensitivity assays

Sensitivity assays were carried out in metal restricted and overload conditions. EDTA (200µM) was used for metals restricted conditions. For metal overload Iron (200 µM), Copper (200µM), Calcium (200mM), Sodium (M), Potassium (1M) and Sodium Dodecyl Sulphate (0.025%) were used. Ethylenediamine tetraacetic acid (EDTA), Ferric Chloride, Cupric Sulphate, Calcium chloride, Sodium Chloride and Sodium Dodecyl Sulphate (SDS) were purchased from Qualigens Fine Chemicals, Mumbai, India. Stock solutions were made in water. *M. grisea* B157 and transformants were grown in minimal agar/ YEG plates supplemented with the required concentrations for 7 days at R.T. Dry weight was also estimated after growth in minimal / YEG media for 7 days.

Sensitivity assays with different drugs were also carried out. The drugs cycloheximide, camptothecin, methotrexate, miconazole and resveratrol were purchased from Sigma Chemical, St. Louis, MO, USA. Stock solutions were made in DMSO. *M. grisea* B157 and transformants were grown in YEG agar plates supplemented with the required drug concentrations for 7 days at R.T. Dry weight was also estimated after growth in minimal/YEG media for 7 days.

### 2.32 Cellular lipid staining by Nile Red

Lipid droplets in germinating conidia and appressoria were visualized by staining with a Nile Red solution consisting of 50mM Tris/maleate buffer, pH 7.5; polyvinylpyrrolidone (20mg/ml ) and Nile Red (2.5µg/ml ) (Sigma Chemical, St. Louis, MO, USA). In all cases, suitable material (germinating conidia and appressoria) were mounted directly in the Nile Red staining solution. Within a few seconds, lipid droplets began to fluoresce when viewed with an epifluorescence microscope at 400X magnification (Olympus BX51, Olympus corporation, Tokyo, Japan) (Thines *et al.*, 2000).

## 2.33 Protoplast production assay

*M. grisea* mycelia were cultured in complete media for 2 days and harvested by centrifugation for 10min at 5,000X g. Mycelia were washed twice and resuspended in 20% sucrose. Lysing enzymes (2mg/ml) (Sigma Chemical, St. Louis, MO, USA) was added to a mycelial suspension. The lysis was stopped after 30, 60, and 90min of incubation by placing the reaction tube on ice. The protoplasts were separated from the mycelia by filtration through Miracloth (Calbiochem, La Jolla, CA, USA). Protoplast release and cell wall degradation were examined using bright field microscopy and number of protoplasts formed was counted using a hemacytometer with triplicates. **(**Jeon *et al.*, 2008)

## 2.34 X–ray fluorescence spectroscopy

The knock-down transformants and wild type strain were grown on complete media at $28^0$C and harvested after 3 days. Fungal biomass was dried at $55^0$C for 24h. Dried biomass was crushed to powder using mortar and pestle. Approximately, 0.05g of powder was used for X-

ray fluorescence spectroscopic analysis (X-ray Fluorescence Spectrophotometer, EDX-800, Shimadzu).

## 2.35 Preparation of dsRNA and siRNA

A fragment of ~160-200 bp was selected from the genes to be silenced. Primers were designed with T7 promoter sequence appended to the 5` end of the primers. Primers were designed to get sense and antisense fragment separately. List of the primers is given in **Table 3**. Two separate PCRs were carried out for sense and antisense fragments. The sense and antisense amplicons were purified using PCR purification columns. Separate *in vitro* transcription reactions were carried out for both sense and antisense fragments according to the manufacturer's instructions. HiScribe T7 *In Vitro* transcription kit (E2030S, New England Biolabs) was used for the *in vitro* transcription reactions. Transcripts were checked on the agarose gel. The two transcription reactions were mixed and incubated at $75^0$C for 5 min and then left on bench to cool to room temperature. dsRNA was purified and DNase treatment was carried out for 1 hour. Again the dsRNA was purified, quantitated and checked on agarose gel. siRNA were prepared from the dsRNA using Shortcut RNase III (M0245S, New England Biolabs) according to the manufacturer's instructions. The siRNA were checked on 12 % PAGE and quantitated using spectrophotometer.

## 2.36 Protoplasts transformation of *M. grisea*

*M. grisea* spores were inoculated in liquid complete medium for 48 hrs at $30^0$C. The resulting mycelium was washed with sterile water and transferred to 1.2 M sorbitol. The

cell wall digestion was performed at $28^0$C with orbital shaking at 80 rpm for 3-4 hrs using Lysing enzymes (L1412, Sigma) 1mg/ml. The protoplast were then separated from non-digested mycelium using cheese cloth and harvested by centrifugation at 4000 rpm for 5 min. The protoplasts were resuspended ($10^6$/ml) in STC (1.2 M sorbitol, 50 mM Tris base, 50 mM $CaCl_{2.}$ $2H_2O$, pH 7.5) buffer and incubated with siRNA for 15 min at $4^0$C. PTC (STC + PEG3350 40%) was added to the mixture and incubated at room temperature for 20 min. Then CMS (complete medium with 1.2M sorbitol) was added to the mixture and incubated at $28^0$C, 80 rpm for 2 hrs. Then the protoplasts were plated on the selection plates with the molten regeneration medium (CM-1%LMA). Colonies were counted after 3-4 days. Fluorescent microscopy was performed for GFP cells.

## 2.37 Transmembrane span prediction

Protein subcellular location prediction was done using Wolf PSORT (Horton *et al.*, 2007). Prediction of membrane spanning domains was implemented using the HMMTOP program of Tusn´ady and Simon (1998) and default parameters. The HMMTOP transmembrane topology prediction server predicts both the localization of helical transmembrane segments and the topology of transmembrane proteins.

| | | | |
|---|---|---|---|
| **Mgg_07771** | Sense | FP | TAA TAC GAC TCA CTA TAG G **ACGATCCCGCCAAGGCCA** |
| | | RP | **ACCCTCTACCTCGACGCGC** |
| | Antisense | FP | TAA TAC GAC TCA CTA TAG G **ACCCTCTACCTCGACGCGC** |
| | | RP | **ACGATCCCGCCAAGGCCA** |
| **Mgg_00551** | Sense | FP | TAA TAC GAC TCA CTA TAG G **GAAGCAGAATGATATCTCAC** |
| | | RP | **TTGGCTGATCAGTGGTACTA** |
| | Antisense | FP | TAA TAC GAC TCA CTA TAG G **GGCTGATCAGTGGTACTAGAA** |
| | | RP | **GAATGATATCTCACTGCTTCA** |
| **Mgg_02876** | Sense | FP | TAA TAC GAC TCA CTA TAG G **AGCAAGCCCTGGGACGTG** |
| | | RP | **GGCGTTCATGTCGACCCT** |
| | Antisense | FP | TAA TAC GAC TCA CTA TAG G **GGCGTTCATGTCGACCCT** |
| | | RP | **AGCAAGCCCTGGGACGTG** |
| **Mgg_11608** | Sense | FP | TAA TAC GAC TCA CTA TAG G **AAGCGCAGCGGAAGCTGT** |
| | | RP | **TTGCTCCAGTCGAGAACCAGA** |
| | Antisense | FP | TAA TAC GAC TCA CTA TAG G **TTGCTCCAGTCGAGAACCAGA** |
| | | RP | **AAGCGCAGCGGAAGCTGT** |
| **Mgg_05790** | Sense | FP | TAA TAC GAC TCA CTA TAG G **GTGGCCTTTAACGAGGAGAA** |
| | | RP | **TTTTGGATGGCCAGGTAGAT** |
| | Antisense | FP | TAA TAC GAC TCA CTA TAG G **TGTTTTGGATGGCCAGGTAGA** |
| | | RP | **GTGGCCTTTAACGAGGAGAA** |
| **eGFP** | Sense | FP | TAA TAC GAC TCA CTA TAG G **AAGTTCATCTGCACCACCG** |
| | | RP | **TTGAAGAAGATGGTGCGCT** |
| | Antisense | FP | TAA TAC GAC TCA CTA TAG G **TTGAAGAAGATGGTGCGCT** |
| | | RP | **GAAGTTCATCTGCACCACCG** |

**Table 3:** List of the primers for preparation of dsRNA and siRNA of 5 laccases and GFP