

## Supplementary File S1: Methodology and Table S1

### Hydrogen Sulfide, Ethylene and Nitric Oxide Regulate Redox Homeostasis and Protect Photosynthetic Metabolism Under High Temperature Stress in Rice Plants

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#### *Chlorophyll Fluorescence Measurement*

Fully expanded leaves were allowed to adapt under dark for 30 min before chlorophyll fluorescence measurements using Junior-PAM chlorophyll fluorometer (Heinz Walz, Germany) were made. Actual pigment system (PS) II, maximal efficiency of PSII, intrinsic PSII efficiency, photochemical quenching, non-photochemical quenching and electron transport rate were calculated. The details of the procedure are given in Supplementary File S1. Minimal fluorescence ( $F_o$ ) and maximum fluorescence ( $F_m$ ) were measured in dark-adapted leaves with a low measuring beam at a light intensity of  $125 \mu\text{mol m}^{-2} \text{s}^{-1}$ , whereas under light-adapted conditions, minimal fluorescence ( $F_o'$ ) and maximum fluorescence ( $F_m'$ ) were measured in the same leaves with saturating light intensity ( $720 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) together with steady-state fluorescence ( $F_s$ ). The variable fluorescence ( $F_v$  and  $F_v'$ ) was calculated using the values of  $F_m - F_o$  and  $F_m' - F_o'$ , and actual PSII efficiency ( $\Phi \text{ PSII}$ ) was determined as  $F_m' - F_s / F_m'$ , maximal efficiency of PSII by using  $F_v / F_m$  and intrinsic PSII efficiency of PSII by using  $F_v' / F_m'$ . Using fluorescence parameters determined in both the light- and dark-adapted states, the photochemical quenching ( $q_P$ ) and non-photochemical quenching (NPQ) parameters were calculated. Photochemical quenching was calculated as  $(F_m' - F_s) / F_v'$  and NPQ as  $(F_m - F_m') / F_m'$  (Maxwell and Johnson, 2000). Electron transport rate (ETR) was calculated by the following formula:  $\Phi \text{ PSII} \times \text{photosynthetic photon flux density} \times 0.5 \times 0.84$  as suggested by Krall and Edwards (1992).

#### *Measurement of Hydrogen Peroxide and Lipid Peroxidation*

The assay of  $\text{H}_2\text{O}_2$  was done following the method of Okuda et al. (1991). Fresh leaf tissues (500 mg) were homogenized in ice-cold 200 mM perchloric acid ( $\text{HClO}_4$ ), followed by centrifugation at  $1200 \times g$  for 10 min. Later the supernatant was neutralized with 4 M KOH. Homogenate was further centrifuged at  $500 \times g$  for 3 min for the removal of insoluble potassium perchlorate. The reaction mixture (1.5 mL) contained 1

mL of the eluate, 80  $\mu$ L of 3-methyl-2-benzothiazoline hydrazone, 400  $\mu$ L of 12.5 mM 3-(dimethylamino) benzoic acid in 0.375 M phosphate buffer (pH 6.5), and 20  $\mu$ L of peroxidase (0.25 unit). The reaction was initiated with the addition of peroxidase at 25°C. The increase in absorbance was estimated at 590 nm on a spectrophotometer.

Lipid peroxidation was estimated by measuring the content of thiobarbituric acid reactive substance (TBARS) as described by Dhindsa et al. (1981). Fresh leaf samples (500 mg) were ground in 0.25% 2-thiobarbituric acid (TBA) in 10% trichloroacetic acid (TCA) using mortar and pestle. The mixture was heated at 95°C for 30 min and rapidly cooled on ice bath. Then, mixture was allowed to centrifuge at  $10,000 \times g$  for 10 min. To one mL of the supernatant 4.0 mL of 20% TCA containing 5% TBA was added. The absorbance of the supernatant was read at 532 nm and corrected for non-specific turbidity by subtracting the absorbance of the same at 600 nm. The content of TBARS was calculated using the extinction coefficient ( $155 \text{ mM}^{-1} \text{ cm}^{-1}$ ).

#### *Assay of Antioxidant Enzymes*

Fresh leaf tissue (200 mg) was collected from the third leaves and ground rapidly in ice-cold extraction buffer containing potassium-phosphate buffer (100 mM, pH 7.0), 0.05% (v/v) Triton X-100 and 1% (w/v) PVP. The homogenates were centrifuged at  $15,000 \times g$  for 20 min at 4°C. The clear supernatant obtained after centrifugation was used for the assay of enzymes. For the assay of ascorbate peroxidase (APX), the extraction buffer was supplemented with 2 mM ascorbate. Protein was estimated according to the protocol described by Bradford (1976), utilizing Bovine serum albumin as a protein standard.

Superoxide dismutase (SOD) activity was determined according to Beyer and Fridovich (1987) and Giannopolitis and Ries (1977) which is based on inhibition of the photochemical reduction of nitro blue tetrazolium (NBT). Five mL of reaction mixture containing 5.0 mM HEPES (pH 7.6), 0.1 mM EDTA, 50 mM  $\text{Na}_2\text{CO}_3$  (pH 10.0), 13 mM methionine, 0.025% (v/v) Triton X-100, 63  $\mu$ mol NBT, 1.3  $\mu$ mol riboflavin and the enzyme extract was illuminated for 15 min ( $360 \mu\text{mol m}^{-2} \text{ s}^{-1}$ ). A control set was also illuminated to correct for background absorbance. A unit of SOD was defined as the amount of enzyme that inhibited the NBT reduction by 50% at 560 nm.

The ascorbate peroxidase (APX) activity was determined according to the method of Nakano and Asada (1981). The assay mixture (1.0 mL) contained phosphate buffer (50 mM, pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbate, 0.1 mM  $\text{H}_2\text{O}_2$ , and enzyme extract. Final volume was observed at 290 nm for 1 min using spectrophotometer. A decrease in absorbance was observed as soon as the reaction was started by adding

H<sub>2</sub>O<sub>2</sub>. APX activity was calculated with the extinction coefficient of 2.8 mM<sup>-1</sup> cm<sup>-1</sup>. One unit of enzyme is the amount necessary to decompose 1 µmol of substrate per min at 25°C.

Glutathione reductase (GR) activity was determined following the method of Foyer and Halliwell (1976) by monitoring the glutathione (GSH) dependent oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm. The reaction mixture (3.0 mL) contained phosphate buffer (25 mM, pH 7.8), 0.5 mM GSSG, 0.2 mM NADPH, and the enzyme extract. The reaction started with the addition of GSSG, and the absorbance showed a decreasing trend. The activity of GR was calculated by using the extinction coefficient of (6.2 mM<sup>-1</sup> cm<sup>-1</sup>). One unit of enzyme is the amount necessary to decompose 1 µmol of NADPH min<sup>-1</sup> at 25°C.

#### *Ethylene Evolution*

The evolution of ethylene in leaves was measured using a gas chromatograph, as described previously by Fatma et al. (2021a). The details are given in Supplementary File S1. Leaf samples were cut into small pieces and placed in 30 mL tubes containing moist paper to prevent evaporation from the tissue, which were then sealed with secure rubber caps and exposed to light for 2 h under the same conditions as for plant growth. With a hypodermic syringe, a 1 mL gas sample was taken from the tubes and analyzed on a gas chromatograph (Nucon 5700, New Delhi, India) equipped with a 1.8 m Porapack N (80–100 mesh) column, a flame ionization detector, and a data station. As a carrier gas, nitrogen was used. Nitrogen, hydrogen, and oxygen flow rates were 30, 30, and 300 mL min<sup>-1</sup>, respectively. The temperature of the detector was set at 150°C. Based on the retention time, ethylene was detected and measured by comparing peaks from standard ethylene concentrations.

#### *RNA Isolation and cDNA Synthesis*

Total RNA was isolated from rice leaves using TRIzol reagent (Ambion, Life Technologies, USA) according to the manufacturer's instructions. With the help of a Nanodrop spectrophotometer (Thermo Scientific, USA), the extracted RNA was quantified. To ensure the integrity of the RNA, each sample was run on agarose formaldehyde gel Turano et al. (1997). The first-strand of the cDNA was made from 1 µg of total RNA of control and treated samples. The cDNA template was synthesized using the reaction mixture containing 20 U/µL Moloney murine leukemia virus reverse transcriptase (MuMLV) enzyme (Fermentas, USA) at 42°C for 50 min and at 70°C for 10 min. The reverse transcription reaction was carried out using 2.5 µM Oligo (dT) 18 primer (Fermentas, USA) and 10 mM dNTPs. Primers for gene

expression analysis were designed using online primer designing software (IDT) and cDNA sequences of selected genes were obtained from NCBI.

#### *Quantitative Real-Time PCR Analysis*

Real-time PCR (RT-PCR) was performed in 96-well reaction plate (Roche, Germany) containing 20 µL reaction mixture of × 10 reaction buffer, 2 mM dNTPs, 1 mM MgCl<sub>2</sub>, 0.35 µM each of forward and reverse primers, 1 µL Sybr green (×10), 10 µg cDNA template and 5 U Taq polymerase on a thermal cycler (Light cycler 480 II, Roche, Germany). All quantifications were normalized to actin DNA fragment amplified by β-actin forward and β-actin reverse primers. The actin gene was used as an internal control for evaluating the efficiency of RT-PCR for particular genes. PCR cycling conditions were as follows: denaturation at 95°C for 3 min, 40 cycles of 95°C (20 s), 66°C (1 min) and 72°C (1 min) with 5 min of final extension at 72°C. The amplified product was resolved on 1.2% agarose gel. The specificity of amplicons was verified by melting curve analysis (60 to 95°C) after 40 cycles. All reactions were performed in three biological replicates (with three technical replicates of each), using gene-specific primers and actin primers as an internal control. The data were taken as the expression of the gene of interest in relation to the internal control in the treated sample compared with the untreated control.

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**Table S1.** Primer pairs used for quantitative RT-PCR

S.No.	Gene	Forward primer	Reverse primer
1	<i>psbA</i>	ATATTGTGGCCGCTCAT	TCCGTTTAGATTGAAAGCCATAG
2	<i>psbB</i>	GCCGGAACATATGTGGTATG	GACCAAGCTTCTGATAAACTGAG
3	<i>Mn-SOD</i> (manganese-superoxide dismutase)	CAAGGATCTGGATGGGTG	CAGGTAGTACGCATGCTC
4	<i>Fe-SOD</i> (iron-superoxide dismutase)	TATACGCTCAGCATTATCACTTT	AACATACATTGCCTGTCA
5	<i>Cu-SOD</i>	GCTAATATCCATGTTGTTGACAG	GAAGTCCGATGATCCCG
6	<i>APX</i> (thylakoid-bound ascorbate peroxidase)	TGATCATCTTAGGGAGGTATTCTACA	TGATTGCCCTCCAGGTTC
<b>Reference gene primer sequences used for quantitative RT-PCR</b>			
1	Actin	GACTGCCAAGACCAGCTCC	CTTCCTAATATCCACGTCGCAC