Expression of antioxidant defense genes in mung bean (*Vigna radiata* L.) roots under water-logging is associated with hypoxia tolerance

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Abstract This study was conducted to examine the extent of oxidative stress and the role of antioxidant enzymes on hypoxia tolerance in highly tolerant wild species *Vigna luteola*, and mung bean (*Vigna radiata*) cvs. T 44 (tolerant) and Pusa Baisakhi (susceptible). Two days of water-logging caused about 40–50% decline in superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) contents in all the genotypes, however, further water-logging to 8 days caused significant increase in $O_2^{\cdot-}$ and H_2O_2 contents, and the values were 80–90% of the control values. In control and revived plants $O_2^{\cdot-}$ and H_2O_2 contents were higher in Pusa Baisakhi, while under water-logging stress T 44 and *V. luteola* showed greater increases in the $O_2^{\cdot-}$ and H_2O_2 contents. Hypoxia induced increase in superoxide dismutase, ascorbate peroxidase, and glutathione reductase activities were higher in T 44 and *V. luteola* compared with Pusa Baisakhi; and the increases in T 44 and *V. luteola* continued up to 8th day of water-logging, while in case of Pusa Baisakhi, the maximum increase was observed only on the 2nd day of water-logging. Gene expression studies showed enhanced expression of *cytosolic-Cu/Zn-superoxide dismutase (SOD)* and *cytosolic-ascorbate peroxidase (APX)* in the roots of waterlogged *V. luteola* and T 44, while little expression was observed in control or treated

plants of Pusa Baisakhi. PCR band products were cloned and sequenced, and partial cDNAs of *Cu/Zn-SOD* and *APX*, respectively, were obtained. Results suggest that increase in the activity of antioxidant enzymes is to scavenge reactive oxygen species produced both during and after relief from water-logging stress.

Keywords Anoxia · Ascorbate peroxidase · Gene expression · Glutathione reductase · Hypoxia · Mung bean · Reactive oxygen species · Superoxide radical · Superoxide dismutase · Water-logging

Introduction

Lack of oxygen or hypoxia and its complete absence or anoxia is a common environmental challenge, which plants have to face throughout their life. Seed imbibitions, flood irrigation, floods, and excess of rainfall are examples of natural conditions leading to root hypoxia or anoxia. A decrease in adenylate energy charge (ATP/ATP + ADP + AMP), cytoplasmic acidification, anaerobic fermentation, elevation in cytosolic Ca^{2+} concentration, changes in the redox state [NAD(P)H/NAD(P)], and a decrease in the membrane barrier function are the main features caused by lack of oxygen (Richard et al. 1994; Crawford and Braendle 1996; Drew 1997; Tadege et al. 1999; Sairam et al. 2008). Regulation of anoxic/hypoxic metabolism is complex and not all the features are well elucidated.

Excessive generation of reactive oxygen species (ROS), or oxidative stress is an integral part of many stress situations, including hypoxia. Hydrogen peroxide accumulation under hypoxic conditions has been shown in the roots and leaves of *Hordeum vulgare* (Kalashnikov Ju et al.

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1994) and in wheat roots (Biemelt et al. 2000). The presence of H_2O_2 in the apoplast and in association with the plasma membrane has been visualized by transmission electron microscopy under hypoxic conditions in four plant species (Blokhina et al. 2001). Recently Kumutha et al. (2009) and Sairam et al. (2009b) have shown that hypoxia induced ROS in pigeon pea roots are due to induction of membrane linked NADPH oxidase. To control the levels of ROS and to protect cells under stress conditions, plant tissues contain several ROS scavenging enzymes viz., superoxide dismutase (SOD: EC1.15.1.1), ascorbate peroxidase (APX: EC1.11.1.11), glutathione reductase (GR: EC 1.6.4.2), and catalase (CAT: EC 1.11.1.6), and a network of low molecular mass antioxidants (ascorbate, glutathione, phenolic compounds, and α -tocopherol). An increase in the activity of antioxidant enzymes in response to environmental stresses have been reported in various studies (Elstner 1986; Bowler et al. 1992; Sairam et al. 2000, 2001, 2002). A 14-fold increase in SOD activity under waterlogged condition has been reported in *Iris pseudacorus* (Monk et al. 1989). The degree of increase in total SOD activity in wheat roots under anoxia was reported to be positively correlated with duration of anoxia (Van Toai and Bolles 1991). Arbona et al. (2008) reported that water-logging induced positive antioxidant response in citrus cultivars *Carrizo citrange* and *Citrumelo* CPB 4475 might be responsible for a higher tolerance to flooding stress, whereas the susceptibility of *Cleopatra mandarin* could be due to less activity of antioxidant enzymes. Wang and Jiang (2007) reported that SOD and APX were mainly involved in water-logging induced antioxidant responses, and the partial water-logging could also significantly affect root antioxidant activities, particularly in water-logging-sensitive cultivar. Similarly, Kumutha et al. (2009) have reported higher activity of SOD, APX, and GR in the water-logging tolerant pigeon pea genotype ICP 301 than susceptible Pusa 207. Increase in the activity of enzymes involved in the ascorbate–glutathione cycle (APX, MDHAR, DHAR, and GR) has also been shown for anaerobically germinated rice seedlings and roots of wheat seedlings (Ushimaru et al. 1997, Albrecht and Wiedenroth 1994).

Mung bean, an important summer–rainy season pulse crop, is exposed to water-logging condition during germination and early vegetative growth phases. Very little physiological and molecular studies have been done on the expression of antioxidant defense genes under water-logging. The objective of this study was to characterize the extent of ROS production during and after water-logging, to validate the role of antioxidant enzymes in water-logging tolerance, and to clone the partial cDNA/genes of *SOD* and *APX* for their subsequent use in developing full length genes.

Materials and methods

Plant material and growth conditions

An experiment was conducted with two cultivated mung bean (*V. radiata* (L.) Wilczek) cvs. T 44 (tolerant) and Pusa Baisakhi (susceptible), and highly tolerant wild genotype *Vigna luteola* under pot-culture condition to study their response to water-logging stress. *V. luteola*, T 44 and, Pusa Baisakhi were obtained from National Bureau of Plant Genetic resources, New Delhi, India, Directorate of Pulse Research, Kanpur, Uttar Pradesh, India and Division of Genetics, Indian Agricultural Research Institute, New Delhi, India, respectively. Sowing was done in 30×30 cm (h $\times$ dia) earthen pots filled with clay-loam soil and compost in 3:1 ratio during the summer–rainy season. Pots were supplied with basal dose of 25 kg ha^{-1} nitrogen and 60 kg ha^{-1} each of phosphorus and potassium. Before sowing, seeds were treated with the required *Rhizobium* culture. Initially four plants were sown in each pot, which were thinned to two plants per pot after 20 days. Water-logging treatment was given by placing pots with 25 days old plants in plastic troughs measuring $100 \times 70 \times 35$ cm (l $\times$ b $\times$ h), and filled with water to a height just 1–2 cm below the soil level in pots. Treatments consisted of control, 2, 4, 6 and 8 days of water-logging, and recovery after 4 days of termination of treatment for 6-day waterlogged plants, as 8 days of water-logging in Pusa Baisakhi (susceptible genotype) resulted in more than 75% mortality during recovery. At each stage samples were collected in quadruplicate from four pots. Observations were recorded on superoxide radical, hydrogen peroxide contents, and activity of superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR), and expression and cloning of *Cu/Zn-SOD* and *APX*. The values are means of four replicates assayed twice ($n = 8$). The design of the experiment was complete randomized (CRD) and data was analyzed by factorial CRD.

Assay of oxidative stress

Superoxide radical content was quantified by its capacity to reduce nitroblue tetrazolium chloride (NBT) and the absorption of end product measured at 540 nm. One gram root tissue was homogenized in 10 mL of pre-cooled phosphate buffer (0.2 M, pH 7.2) containing 1 mM diethyl dithio carbamate (DEDC), to inhibit SOD activity. The homogenate was centrifuged in Sigma refrigerated centrifuge (model 3 K 30, Osterode, Germany) at $10,000 \text{ g}$ for 10 min and the supernatant was used immediately for the estimation of superoxide radical (Chaitanya and Naithani 1994). The reaction mixture contained 0.25 mL tissue extract, 0.075 mM NBT, 25 mM Na_2CO_3 , 0.1 mM EDTA,

13.33 mM L-methionine and water to make the volume 3 mL. Reaction mixtures were incubated at 30°C for 10 min and absorbance was recorded at 540 nm. Superoxide radical content was calculated according to its extinction coefficient $\varepsilon = 12.8 \text{ mM}^{-1} \text{ cm}^{-1}$.

Hydrogen peroxide was estimated by forming titanium–hydro peroxide complex (Rao et al. 1997). One gram root material was ground with liquid nitrogen and the fine powdered material was mixed with 10 mL cooled acetone in a cold room (10°C). Mixture was filtered with Whatman No.1 filter paper followed by the addition of 4 mL titanium reagent and 5 mL ammonium solution to precipitate the titanium–hydro peroxide complex. Reaction mixture was centrifuged at 10,000 g for 10 min in the Sigma refrigerated centrifuge (model 3 K 30, Osterode, Germany). Precipitate was dissolved in 10 mL of 2 M H_2SO_4 and then recentrifuged. Supernatant was read at 415 nm against blank in UV–visible spectrophotometer (model Specord Bio-200, AnalytikJena, Germany).

Preparation of enzyme extract

Enzyme extract for SOD, APX, and GR was prepared by freezing 1 g root samples in liquid nitrogen, followed by grinding with 5 mL extraction buffer consisting of 0.1 M phosphate buffer, pH 7.5, containing 0.5 mM EDTA in case of SOD and GR or 0.1 M phosphate buffer, pH 7.5 containing 0.5 mM EDTA and 1 mM ascorbic acid in case of APX. Brie was passed through four layers of cheesecloth and filtrate was centrifuged for 20 min at 4°C and 21,000 g, and the supernatant was used as enzyme extract.

Antioxidant enzymes estimation

Superoxide dismutase activity was assayed by following the enzyme mediated decrease in absorbance of formazone formed by superoxide anion with NBT (Dhindsa et al. 1981). The reaction mixture contained 13.33 mM methionine, 25 mM NBT, 0.1 mM EDTA, 50 mM potassium phosphate buffer (pH 7.8), 50 mM sodium carbonate and 0.1 mL enzyme. Reaction was started by adding 2 μM riboflavin and placing the tubes under two 15 W fluorescent lamps for 15 min. To distinguish SOD isoforms viz., Cu/Zn-SOD,

Fe-SOD and Mn-SOD, the sensitivity of Cu/Zn-SOD to cyanide (3 mM), and Cu/Zn-SOD and Fe-SOD to hydrogen peroxide (5 mM) were used, whereas Mn-SOD is unaffected (Yu and Rengel 1999). A complete reaction mixture without enzyme, which gave the maximal color, served as a blank. Reaction was stopped by switching off the light and putting the tubes into dark. A non-irradiated complete reaction mixture served as a reference. The absorbance was recorded at 560 nm, and 1 unit of enzyme activity was taken as that amount of enzyme, which reduced the absorbance reading to 50% in comparison with tubes lacking enzyme (blank).

Ascorbate peroxidase activity was assayed by recording the decrease in absorbance due to enzyme induced oxidation of ascorbic acid at 290 nm (Nakano and Asada 1981). The 3 mL reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA, 1.5 mM H_2O_2 and 0.1 mL enzyme. The reaction was started with the addition of hydrogen peroxide. Absorbance was measured at 290 nm in a UV–visible spectrophotometer (model Specord Bio-200, AnalytikJena, Germany).

Glutathione reductase activity was assayed as per the method of Smith et al. (1988). The reaction mixture contained, 66.67 mM potassium phosphate buffer (pH 7.5) and 0.33 mM EDTA, 0.5 mM DTNB (in 0.01 M potassium phosphate buffer, pH 7.5), 66.67 μM NADPH, 666.67 μM oxidized glutathione (GSSG), 0.1 mL enzyme extract. Reaction was started by adding GSSG. The increase in absorbance at 412 nm was recorded spectrophotometrically.

Gene expression by RT-PCR

Nucleotide sequences for candidate genes were obtained from National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). The Basic Local Alignment Search Tool (Altschul et al. 1997) (<http://www.ncbi.nlm.nih.gov/BLAST/>) was used to identify the homologs of candidate genes. For RT-PCR expression analysis and cloning of cDNAs, the following oligonucleotide primers were designed manually, and oligo quality (to avoid primer dimer, self dimer etc.), GC % and T_m were analyzed by using Oligoanalyzer 3.0 tool (<http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/>, Integrated DNA Technologies, Coralville, IA 52241, USA).

Primer Name	Sequence	GC content (%)	T_m (°C)	Amplicon size (bp)
Tubulin-F	CTTGACTGCATCTGCTATGTTTCAG	45.8	55.5	422
Tubulin-R	CCAGCTAATGCTCGGCATACTG	54.5	58.4	
APX-F	CTCAGAGGYTTMATCGCTGAGAAG	45.7	64.6	689
APX-R	CAGCAAACCCAAGCTCRGARAGC	49.7	66.3	
Cu/Zn-SOD-F	ATGGTKAAGGCTGTGGCAGTTC	52.3	58.9	408
Cu/Zn-SOD-R	ACCTTTCCCAAGATCATCAGGAGGATC	45.8	56.5	

For gene induction studies 25 days old plants were subjected to water-logging treatment for 24 h, as 48 h water-logging treatment resulted in decline in gene expression of *Cu/Zn-SOD* and *APX* in all the genotypes. Root samples were harvested from control and treated plants. Total RNA from the root tissue was extracted using Trizol reagent (GibcoBRL) as per the recommendations of the manufacturer. DNA contamination was removed from the RNA samples using DNase I (Qiagen Science, Maryland, USA). One microgram of total RNA was reverse transcribed using gene specific degenerate primers and Qiagen one step RT-PCR kit. PCR conditions were standardized using gene-specific primers for tubulin. Linear amplification for semi-quantitative RT-PCR was obtained with 35 cycles. Reactions were conducted using My Genie 32 Thermal Block PCR (Bioneer, Korea) under the following conditions: initial PCR activation step, 15 min at 95°C; reverse transcription, 30 min at 50°C; denaturation, 1 min at 94°C; annealing, 1 min at 60°C; extension, 1 min at 72°C; final extension, 10 min at 72°C. The amplification products were electrophoresed on 1.2% agarose gel at 120 V in TBE buffer (0.4 M Tris–borate, 0.001 M EDTA, pH 8.0) using known concentration DNA ladders. Gels were stained with ethidium bromide and visualized on Uvi Pro Gel Documentation system (Uvitec, England).

Gene sequencing

RT-PCR amplified cDNAs were fractionated on agarose gel and purified. The purified cDNAs for each gene were cloned into pTz57R/T vector and transformed into *E. coli* (strain DH5 α) cells. DH5 α cells transformed with recombinant plasmids were selected based on antibiotic resistance as well as α -complementation method. Ampicillin resistant putative recombinants were selected for further analysis. Plasmid were isolated from the confirmed colonies and restriction analysis was carried out by using *Kpn*I and *Hind*III enzymes flanking the cloning site of the vector pTz57R/T, to confirm the presence of cloned insert cDNA. Cloned insert cDNA in the pTz57R/T vector was sequenced by dideoxy chain termination method (Sanger et al. 1977) using T7 and SP6 primers.

Results

ROS contents

Two days after water-logging total superoxide radical ($O_2^{\cdot-}$) content declined over control plants in all the genotypes (Fig. 1a). However, continuous water-logging up to 8 days resulted in significant increase in total $O_2^{\cdot-}$ production over 2-day waterlogged plants in the three

genotypes, though remaining less than the control plants. The increase was greater in *V. luteola* and T 44 than in Pusa Baisakhi. Recovery recorded after 4 days of termination of treatment resulted in a drastic increase in $O_2^{\cdot-}$ content over that observed in waterlogged and control plants of the three genotypes, and the increase in $O_2^{\cdot-}$ production was highest in Pusa Baisakhi compared to *V. luteola* and T 44.

Hydrogen peroxide content showed pattern similar to $O_2^{\cdot-}$ content. In control and recovered plants H_2O_2 content was higher in Pusa Baisakhi than in *V. luteola* and T 44 (Fig. 1b). Two days of water-logging resulted in significant decline in hydrogen peroxide content in the three genotypes compared to that observed in control plants. However, further water-logging up to 8 days resulted in significant increase in H_2O_2 content compared to 2-day waterlogged, but remained lower than control plants in all the genotypes. During the water-logging phase *V. luteola* and T 44 showed higher H_2O_2 content than observed in Pusa Baisakhi. Recovery resulted in a drastic increase in H_2O_2 content over that observed in control and water-logged plants in the three genotypes, and Pusa Baisakhi showed significantly higher H_2O_2 content than *V. luteola* and T 44 (Fig. 1b).

Enzyme activities

Water-logging caused significant increase in the activity of SOD and its isozymes in *V. luteola* and T 44 up to 8th day of stress, while in Pusa Baisakhi a slight increase was

Fig. 1 Effect of water-logging on total superoxide radicals (a) and hydrogen peroxide content (b) in root tissues in *V. luteola* and *V. radiata* genotypes T 44 (tolerant) and Pusa Baisakhi (susceptible). LSD significant ($P \leq 0.05$). Vertical bars show $\pm$ SE of mean

Fig. 2 Effect of water-logging on the activity of total superoxide dismutase (SOD) (a), Cu/Zn-SOD (b), Mn-SOD (c) and Fe-SOD (d) in root tissues in *V. luteola* and *V. radiata* genotypes T 44 (tolerant) and Pusa Baisakhi (susceptible). LSD significant ($P \leq 0.05$). Vertical bars show $\pm$ SE of mean

observed only on 2nd day of water-logging and thereafter, the activity decreased with duration of stress (Fig. 2a). During recovery the SOD activity slightly decreased in *V. luteola* and T 44 and increased in Pusa Baisakhi, however, the activity in *V. luteola* and T 44 was 445 and 381% higher than observed in Pusa Baisakhi. Water-logging induced increase in total SOD activity in *V. luteola* and T 44 which was mainly due to the increase in Cu/Zn-SOD, which contributed to about 51, 54, 58, 59% and 57, 56, 58, 59% at 2, 4, 6 and 8-day water-logging treatment, respectively. Mn-SOD accounted for about 29, 27, 25, 25% and 23, 25, 25, 25% of total SOD activity at 2, 4, 6 and 8-day of water-logging treatment in *V. luteola* and T 44, respectively. Fe-SOD had lesser contribution to total SOD activity. All the isoforms of SOD were greatly reduced at 4,

Fig. 3 Effect of water-logging on the activity of ascorbate peroxidase (a) and glutathione reductase (b) in root tissues in *V. luteola* and *V. radiata* genotypes T 44 (tolerant) and Pusa Baisakhi (susceptible). LSD significant ($P \leq 0.05$). Vertical bars show $\pm$ SE of mean

6 and 8 days of water-logging stress in Pusa Baisakhi (Fig. 2b–d).

There was significant increase in the activities of APX and GR at 2 d of water-logging in all the genotypes (Fig. 3a, b). Further water-logging of 4, 6, and 8 days caused continuous increase in APX and GR activity in *V. luteola* and T 44. However, in case of Pusa Baisakhi, 4-day water-logging showed only slight increase in APX and GR activity, while further water-logging of 6 and 8 days resulted in significant decline in all the enzymes. During recovery, Pusa Baisakhi showed slight increase in the activity of APX, and decline in GR activity, while *V. luteola* and T 44 showed decline in the activity of both the enzymes, however, the activities were many times higher than the control and recovered plants of Pusa Baisakhi.

Gene expression of *Cu/Zn-SOD* and *APX*

Gene expression studies for *Cu/Zn-SOD* and *APX* by RT-PCR elicited bands of about 410 and 690 bp. There was very little constitutive expression of *cyt.-Cu/Zn-SOD* gene (mRNA) in *V. luteola*, T 44, and Pusa Baisakhi. However, 24-h water-logging treatment induced a very significant increase in *cyt.-Cu/Zn-SOD* gene expression only in *V. luteola* and T 44, with only a little increase in Pusa Baisakhi (Fig. 4a). *Cyt-APX* gene showed significant expression in *V. luteola* and T 44 under 24 h water-logging.

In case of Pusa Baisakhi very little *APX* expression was observed both in control and water-logging conditions (Fig. 4b). The tubulin expression was almost constant in both the genotypes, and did not change under control and waterlogged conditions (Fig. 4c).

Sequencing of Cu/Zn-SOD and APX

Cyt.-Cu/Zn-SOD gene specific primers yielded partial gene coding sequences of 399, 406, and 392 bp in *V. luteola*, T 44 and Pusa Baisakhi, respectively. Partial nucleotide sequences for *cyt.-Cu/Zn-SOD* of the three genotypes are given below:

Vigna luteola

AGTAACGAAGTCTCGGGTACTATTAAGTTCAGTCA
GGAGGGAAATGGTCCAACCACTGTAAGTGAAGT
CTTGCTGGTCTTAAGCCTGGCCTCCACGGCTTCCA
TATCCATGCCTTGGGAGACACCACAAACGGTTGC
ATTTCAACTGGACCACATTTCAATCCTAATGGGAA
GGAACATGGTGGCCCTGAGGATGAGACTAGACAT
GCTGGTGATTTAGGAAATATCAATGTTGGTGATGA
TGGAAGTGTAAAGCTTCACCATTACTGACAACCATA

TCCCTCTCACTGGAACAACTCCATCATAGGAAG
GGCTGTTGTTGTCCATGCCGATCCTGATGATCTTG
GGAAAGGTGGTCACGAGCTTAGCAAACTACTGG
AAATGCTGGTGGCAGAGTA

T 44

GTTTAGTAACAGTAACGAAGTCTCGGGTACTATT
AACTTCAGTCAGGAGGGAAATGGTCCAACCACTG
TAACTGGAAGTCTTGCTGGTCTTAAGCCTGGCCTC
CACGGCTTCCATATCCATGCCTTGGGAGACACCAC
AAACGGTTGCATTTCAACTGGACCACATTTCAATC
CTAATGGGAAGGAACATGGTGGCCCTGAGGATGA
GACTAGACATGCTGGTGATTTAGGAAATATCAAT
GTTGGTGATGATGGAAGTGTAAAGCTTCACCATTAC
TGACAACCATATCCCTCTCACTGGAACAACTCCA
TCATAGGAAGGGCTGTTGTTGTCCATGCCGATCCT
GATGATCTTGGGAAAGGTGGTCACGAGCTTAGCA
AAACTACTGGAAATGCTGGTGGCAGA.

Pusa Baisakhi

AGTTAGTAACAGTAACGAAGTCTCGGGTACTATT
AACTTCAGTCAGGAGGGAAATGGTCCAACCACTG
TAACTGGAAGTCTTGCTGGTCTTAAGCCTGGCCTC
CACGGCTTCCATATCCATGCCTTGGGAGACACCAC
AAACGGTTGCATTTCAACTGGACCACATTTCAATC
CTAATGGGAAGGAACATGGTGGCCCTGAGGATGA
GACTAGACATGCTGGTGATTTAGGAAATATCAAT
GTTGGTGATGATGGAAGTGTAAAGCCGACCATT
CTGGCAACCATATCCCTCTCACTGGAACAACTCC
ATCATAGGAAGGGCTGTTGTTGTCCATGCCGATCC
TGATGATCTTGGGAAAGGTGGTCACGAGCTTAGC
AAACTACTGGAA.

V. luteola, T 44, and Pusa Baisakhi showed 100, 99, 98% similarity with *cyt.-Cu/Zn-SOD* of *Pisum sativum* (GenBank Acc. No. ABQ189165). *V. luteola* showed 99 and 96% similarity with T 44 and Pusa Baisakhi, respectively, while the similarity between T 44 and Pusa Baisakhi was 98%.

Cyt-APX gene specific primers yielded partial gene coding sequences of 662, 630, and 622 bp in *V. luteola*, T 44, and Pusa Baisakhi, respectively. Partial nucleotide sequences for *cyt.-APX* of the three genotypes are given below:

Vigna luteola

TTCATCGCTGAGAAGAGATGCGCTCCTCTGATGCT
CCGTTTGGCATGGCACTCTGCTGGTACCTACGACG

Fig. 4 Expression analysis of **a** cytosolic Cu/Zn-superoxide dismutase (*cyt-Cu/Zn-SOD*), **b** cytosolic ascorbate peroxidase (*cyt.-APX*) and **c** tubulin genes under 24 h water-logging stress and control conditions. Gene expression was determined by RT-PCR utilizing gene specific primer sets for each gene. β -Tubulin gene expression was used as an internal control. (1-1 Kb ladder, 2-Control *V. luteola*, 3-Treated *V. luteola*, 4-Control T 44, 5-Treated T 44, 6-Control Pusa Baisakhi, 7-Treated Pusa Baisakhi)

TTAGCTCGAAGACCGGTGGTCCCTTCGGAACCATG
AAGCACCCCGCTGAACTCGCTCACGGTGCCAACA
ACGGTCTCGATATCGCTGTTAGGCTTTTGGAAACCA
ATTAAGCAGAGTTTCCTATCTTGAGCTACGCAGA
TTTCTACCAGTTGGCTGGCGTTGTCGCAGTTGAGG
TCACTGGTGGACCTGAAGTTCCTTTCCACCCGGGA
AGAGAGGACAAGCCAGAACCACCTCCAGAGGGTC
GCTTGCCCGATGCAACCAAGGGGTCTGATCATCTT
AGGGATGTGTTTCGGCAAAGCTATGGGCCCTTAGTGA
TCAAGATATTGTTGCTCTATCTGGTGGCCACACCA
TTGGAGCGGCACACAAGGAGCGTTCAGGATTTGA
AGGCCCGTGGACCTCAAACCCTCTTATTTTCGACA
ACTCATACTTTAAGGAGTTGTTGAGTGGTGAAAAG
GAAGGCCTCCTTCAGCTGCCTTCTGACAAGGCACT
TTTGTCTGATCCTGTATTCCGCCCTCTTGTGAAAA
ATATGCAGCGGACGAAGATGCCTTCTTCGCTGATT
ACGCAGTTGCTCACCAAAGCTCTCCGAGCTGGG.

T 44

GCATGGCACTCTGCTGGTACCTTTGACGTTAGCAC
GAAGACCGGTGGTCCCTTCGGAACCATCAAGCAC
CCTGCGGAACTCGCTCACGGTGCCAACAACGGTC
TCGACATAGCTGTTAGGCTTTTGGAGCCAATCAAA
GCAGAGTTTCCTATCTTGAGCTACGCAGATTTCTA
CCAGCTGGCTGGCGTTGTTGCAGTTGAGATAACTG
GTGGACCCGAAGTACCTTTTCACCCGGGCAGAGA
GGACAAACCAGAACCACCTCCAGAGGGTGCCTTG
CCTGATGCAACCAAGGGGTCTGATCATCTTAGGG
ATGTGTTTCGGCAAGGCTATGGGGCTTAGTGATCA
GGATATTGTTGCTCTATCTGGTGGTCACACCATCG
GAGCGGCACACAAGGAGCGTTCAGGATTTGAGGG
TCCGTGGACCTCAGACCCTCTTATATTTGACAAC
CACACTTTAAGGAGTTGTTGAGTGGTGAAAAGGA
AGGCCTCCTTCAGCTGCCTTCTGACAAGGCACTTC
TGTCAGATCCCGTATTCCGCCCTCTTGTGAAAAA
TATGCAGCGGACGAAGATGCCTTCTTTGCTGATTA
TGCAGTTGCTCACCAAATGCTCTCCGAGCTAGGGT
TTGACTG.

Pusa Baisakhi

GATTTCTACCAGCTGGCTGGCGTTGTTGCAGTTGA
GATAACTGGTGGACCCGAAGTACCTTTTCACCCG
GGCAGAGAGACTCTGCTGGTACCTTTGACGTTAG
CACGAAGACCGGTGGTCCCTTCGGAACCATCAAG
CACCTGCGGAACTCGCTCACGGTGCCAACAACG
GTCTCGACATAGCTGTTAGGCTTTTGGAGCCAATC
AAAGCAGAGTTTCCTATCTTGAGCTACGCAGACA
AACCAGAACCACCTCCAGAGGGTGCCTTGCTGA
TGCAACCAAGGGGTCTGATCATCTTAGGGATGTG

TTCGGCAAGGCTATGGGGCTTAGTGATCAGGATA
TTGTTGCTCTATCTGGTGGTCACACCATCGGAGCG
GCACACAAGGAGCGTTCAGGATTTGAGGGTCCGT
GGACCTCAGACCCTCTTATATTTGACAACCTCACAC
TTTAAGGAGTTGTTGAGTGGTGAAAAGGAAGGCC
TCCTTCAGCTGCCTTCTGACAAGGCACTTCTGTCA
GATCCCGTATTCCGCCCTCTTGTGAAAAATATGC
AGCGGACGAAGATGCCGTCTTTGCTGATTATGCA
ATTGCTCACCAAAGCTCTCCGAGCTAGGGTTTTGC
TGA.

V. luteola, T 44, and Pusa Baisakhi showed 95, 92, and 93% similarity with the *APX* complete CDS of *V. unguiculata* (GenBank Acc. No. U61379). *V. luteola* showed 92% similarity with T 44 and Pusa Baisakhi, while there was 98% similarity between T 44 and Pusa Baisakhi.

Discussion

We have earlier reported that part of the basis of water-logging tolerance of wild species *V. luteola* and mung bean (*V. radiata*) cv. T 44 could be comparatively greater accumulation of total and reducing sugars, mRNA expression and activity of sucrose synthase and alcohol dehydrogenase in roots, which was manifested in higher relative water content and chlorophyll contents in leaves, and membrane stability index (lower membrane injury) both in roots and leaves than susceptible cv. Pusa Baisakhi (Sairam et al. 2009a, b).

A very striking observation under water-logging is the production of various ROS, especially superoxide radical and hydrogen peroxide (Yan et al. 1996). Though 2 day waterlogged plants showed a decline in $O_2^{\cdot-}$ and H_2O_2 contents as compared to control or untreated plants, from 4th to 8th day there was significant increase in the contents of $O_2^{\cdot-}$ and H_2O_2 . Further comparatively tolerant *V. luteola* and T 44 showed relatively higher $O_2^{\cdot-}$ and H_2O_2 levels compared to Pusa Baisakhi (susceptible cv.) during the water-logging phase as against higher ROS levels in Pusa Baisakhi in control and revived plants. The decline in $O_2^{\cdot-}$ and H_2O_2 production on 2nd day of water-logging could be attributed to a shift from aerobic respiration to fermentation and consequently a blockage in the mitochondrial site of ROS production. Further, the rise in ROS from 4th to 8th day of water-logging treatment could be due to the induction of membrane linked NADPH oxidase. Water-logging induced increase in the activity of diphenylene iodonium chloride-sensitive NADPH oxidase dependent superoxide production as well as increase in the expression of *NADPH oxidase* has been reported in pigeon pea (Kumutha et al. 2009 and Sairam et al. 2009b). Blokhina et al. (2001) reported H_2O_2 formation during anoxia as evidenced by electron dense insoluble precipitate

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