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ARTICLE

A modified method of total RNA isolation and quantitative analysis of superoxide dismutase gene expression from different organs of *Ipomoea carnea*

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ABSTRACT *Ipomoea carnea* (*I. carnea*) has unique biological features for the study of cellular and molecular adaptation mechanisms due to presence of diverse alkaloid and its cadmium tolerance capacity. The present study was directed to quantify total SOD content in different organs of the plant and further extended to relative quantification of cytosolic CuZn-SOD, Fe-SOD and Mn-SOD mRNA. A modified method of total RNA isolation from the plant *I. carnea* which is rich in alkaloids has been described. Total SOD content of apical and lateral bud was highest, but transcript abundance of cytosolic CuZn-SOD was much lower as compared to root and leaves. In these cases Mn- and Fe-SOD mRNA was relatively higher and perhaps that was contributing to the high SOD activity. However, less photosynthetically active organs like root and petal show less SOD activity but mRNA level of cytosolic CuZn-SOD was competitive in these cases. The results showed that SODs in different compartments are differently regulated and each SOD isoenzyme must be performing specific function related to its cellular localization and expression of the protein isoforms depend upon local accumulation of superoxide.

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KEY WORDS

differential expression
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Superoxide dismutase (SOD, EC 1.15.1.1) in plant, a key enzyme in reactive oxygen metabolism, catalyzes the dismutation of $O_2^{\cdot -}$, forming molecular oxygen and H_2O_2 (Fridovich 1975; Bannister et al. 1987). The H_2O_2 thus produced is quickly scavenged by catalase and peroxidase group of enzymes present in the plant cell (Murai and Murai 1996). The individual member of protein in this group is characterized on the basis of the metal ion cofactor they harbour, and as such four different classes like CuZn-SOD, Mn-, Fe-, and Ni- have been reported to date. In higher plants, distinct immunologically distinguishable CuZn-SODs remain distributed in cytosol, chloroplasts, peroxisomes, and in extracellular space. Mn-SOD is housed in mitochondria. Further, the distribution of cuprozinc and mangano SOD in mitochondria is restricted respectively to inter-membrane space and the mitochondrial matrix (Salin and Bridges 1981). The chloroplast, which is devoid of mangano SOD (Salin and Bridges 1981), harbours Fe-SOD in addition to cuprozinc SOD. Ni-SOD has not been reported from plants (Alscher et al. 2002). Based on the criteria of amino acid sequence, spectral characteristics, and three dimensional fold, CuZn-SODs are believed to be evolutionary distinct from Mn and Fe containing SODs (Perry et al. 2010).

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Exposure to photo-inhibitory light condition, ozone fumigation, ultraviolate-B radiation or other environmental biotic and abiotic stresses, including metal toxicity impose oxidative stress with steady formation of $O_2^{\cdot -}$ in plants (Kliebenstein et al. 1998). Nevertheless, expression of different iso-forms SOD can also vary in different tissues depending on the local accumulation of $O_2^{\cdot -}$. The existence of three different isoforms of SOD (CuZn, Mn, and Fe), each of which is typically encoded by small but distinct gene family, further complicate the situation as regard to their specific role in view of tissue localization (Alscher et al. 2002), and more importantly the specificity of metabolic activity endowed by the tissue.

To date, the protective role of SOD in plants has been explored mainly by transgenic approaches, primarily through over-expression or by correlation of SOD expression in different stress condition (Gupta et al. 1993). However, the factors which controls the expression of particular iso-forms of SOD in specific tissues along with its metabolic specificity is still an enigma.

Ipomoea carnea (morning glory), subsp. *fistulosa* (Jacq.) is a toxic weed found abundantly in many tropical countries including India. Presence of various nortropane groups of alkaloids in different organ of plants are identified as potent toxic compound (Ikeda et al. 2003; Hueza et al. 2007). Recently, the plant has also gained much attention due to its suitability in phyto-extraction of cadmium (Cd) from

Table1. Oligonucleotide primers used for RT-PCR and Real time PCR analysis. 'F' and 'R' stands respectively for forward and reverse primers used in the experiments. Full length accession number of the corresponding genes has been mentioned in the table.

Name of the oligo	Accession No.	Sequence (5′ to 3′)	Amplicon length (bp)	T_m
18sF	AK059783	GACTACGTCCCTGCCCTTTGTACAC	128	60°C
18sR		AGGTTCAATGGACTTCTCGCGACGTC		
Cyt CuZn *SOD* F	JQ906095	CGGGCCTTCAAACCTGGTCTT	254	60°C
Cyt CuZn *SOD* R		CATGAACAACAACAGCTCTTCC		
Fe *SOD* F	M55910.1	GCTGCGGCAACACAATTTGGCTCTGG	157	61°C
Fe *SOD* R		AAGTCCAGATAGTAAGCATGCTCCCA		
Mn *SOD* F	JF509743.1	GTACAAGGTTCTGGCTGGGTGTGGCTG	186	61°C
Mn *SOD* R		ACTTCACATGCATATTTCCAGTTCACA		

soil (Ghosh and Singh 2005). The excess of Cd in plant tissue can stimulate the formation of reactive oxygen species (ROS), disturbs the cellular redox balance, suppresses cell expansion, and leads to significant accumulation of H_2O_2, which causes hardening of the cell wall and also activate the formation of phytochelatins and metallothioneins (Metwally et al. 2003; Gill et al. 2013). These cellular events can lead to reprogramming of the antioxidants system in plants, in order to cope with the oxidative stress caused due to the heavy metal toxicity. It is also believed that, SOD plays a dual role in preventing metal toxicity by cleaning the $O_2^{\cdot -}$ radical and preventing the accumulation of free metal (Okamoto and Colepicolo 1998).

Although, SOD has been extensively studied for their role in stress tolerance, development and morphogenesis, organ and tissue specific expression of the different iso-form is lacking in the literature. The objective of the present report is to evaluate the organ specific activity of SOD in a cadmium tolerant plant; *Ipomoea carnea* (*I.carnea*) and further extended to mRNA quantification of Fe, Mn and cytosolic CuZn-SODs. The study also describes a modified method for isolation of high quality total RNA from the plant rich in alkaloids and phenolics.

Materials and Methods

Plant material and reagents

Plant material *I. carnea* was collected from the Institute of Life Sciences campus, Bhubaneswar, India and washed thoroughly with distilled water before use in the experiments.

Unless mentioned, all analytical grade reagents were procured from Sigma. Taq DNA polymerase was obtained from Promega while AffinityScript QPCR cDNA synthesis Kit was from Stratagene. For real time PCR QuantiTect SYBR Green PCR kit from Qiagen was used. All the oligonucleotide primers used in this study were obtained from Ocimum Biosolution, India.

Preparation of crude extract

Crude extract from different organs of mature *I. carnea* plant (200 mg) at flowering stage were prepared by homogenizing in liquid nitrogen and then suspended in 1 ml of ice cold 50 mM K-PO_4 buffer (pH 7.8) containing 0.1 mM Phenylmethanesulfonyl fluoride (PMSF), 8% (w/v) polyvinylpolypyrrolidone (PVPP) and protease inhibitor cocktail (Roche). The homogenate was then centrifuged at 15,000 x g for 30 min at 4°C and the supernatant was used for SOD activity assay. The protein concentration was measured following modified Bradford assay (Zor and Selinger 1996).

SOD activity assay

Spectrophotometric assay of SOD activity was carried out by following (Beauchamp and Fridovich 1971). Assay were performed in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.8), 1.33 mM Diethylenetriamine pentaacetic acid (DTPA), and 2.45 mM Nitro blue tetrazolium salt (NBT), 1.8 mM xanthine and a suitable concentration of xanthine oxidase (till a linear curve with a slope of 0.025 absorbance per min in time scan was obtained). One unit of SOD activity was defined as the amount of protein which produced one half of the maximum competition against NBT in the specified system. The final activity was recorded after deducting out the non-specific SOD like activity produced by many low molecular compounds (Yamahara et al. 1999; Sharma et al. 2004). This was achieved by measuring the activity in samples following heat inactivation of protein (95°C for 20 min).

All assays were done using three different crude extract preparations and five different concentrations of proteins were used and data are the means of three replicates.

RT- PCR primer design

The sequences of the primer used in this study are given in Table 1. Rice 18S rRNA (AK059783) was used as the

housekeeping genes as an internal control. In order to amplify shorter fragment of *I. carnea* SODs, internal primers were designed on the basis of homology based nucleotide sequence alignment (Fig. 1-Cytosolic CuZn-SOD, Fig. 2-Fe-SOD and Fig. 3-Mn-SOD) using ClustalX (Larkin et al. 2007) taking selected plant species for each class of SODs and degenerate primer pairs (Table 1) were designed from the most conserved sequence (shaded with yellow region, Fig. 1, 2 and 3). Annealing temperature of all the primers was kept constant at ~ 60°C.

Isolation of total RNA and cDNA preparation

Isolation of total RNA from different organs of I. *carnea* was carried with some modifications from the protocol described by Natalia et al.,(Kolosova et al. 2004). Prior to isolation of RNA, all the plant material were kept in 0.1% Diethyl pyrocarbonate (DEPC)-treated water for 30-60 min. Since the plant is rich in phenolic and alkaloid compounds, concentration of PVPP was increased to 8% (w/v) in the extraction buffer [200 mM Tris-HCl, pH 8.5, 1.5% lithium dodecylsulfate, 300 mM LiCl, 10 mM disodium salt EDTA, 1% (w/v) sodium deoxycholate, 1% (w/v), Nonidet P-40 (NP-40)]. 5 mM thiourea, 1 mM aurintricarboxylic acid, 10 mM dithiothreitol, and 2% (w/v) PVPP were added to the extraction buffer just before use.

Plant tissue (1 g) was grounded to fine powder in liquid nitrogen using a mortar and pestle and the powder was transferred to a 50-mL polypropylene tube. 20ml of extraction buffer per gram tissue was added and vigorously shaken to uniformly suspend the sample. The suspension was then frozen at -80°C for 1 h and the extracts were centrifuged at 5000×g for 30 min at 4°C. One-thirtieth volume of 3.3 M sodium acetate (pH 6.1) and 0.1 volume 100% ethanol were added to the supernatant, and the mixture was chilled on ice for 10 min to precipitate polysaccharides. Polysaccharides were pelleted by centrifugation at 5000×g for 30 min at 4°C. In order to precipitate nucleic acids, one-tenth volume of 3.3 M sodium acetate and 0.6 volume of ice-cold isopropanol were added to the supernatant, and the solution was left at -80°C for 1 h. Nucleic acid pellets were collected by centrifugation for 45 min at 5000×g at 4°C. The supernatant was removed, and the pellet was resuspended in 2 mL of TE ((10 mM Tris-HCl, pH 8.0, 1 Mm Ethylenediaminetetraacetic acid (EDTA)) and 2 mL 5 M NaCl and kept on ice for 30 min with periodic vortex mixing. The samples were mixed with 4 mL of 10% Cetyltrimethylammonium bromide (CTAB) at room temperature, vortex mixed, and incubated for 5 min at 65°C to remove residual polysaccharides. Mixtures were then extracted twice with an equal volume of chloroform/ isoamylalcohol (24:1, v/v). One-fourth volume of 5 M LiCl was added to the supernatant, mixed, and kept at 4°C overnight. RNA was pelleted by centrifugation at 5000×g for 30 min at 4°C. The supernatant was poured off, and the residual

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CLUSTAL 2.1 multiple sequence alignment-Cytosolic CuZn-SOD

Ipomoea         ATGGTGAAGGCTGTCGCAGTTCTTAGCAGTAGTGAAGGTGTCAGCGGCACCATTTTCTTC 60
Populus         ATGGTGAAGGCTGTAGCTGTTCTTAATAGCAGTGAAGGTGTGAGTGGCACCATCTTCTTT 60
Oryza           ATGGTGAAGGCTGTTGTTGTGCTTGGTAGCAGTGAGATTGTTAAGGGCACTATCCACTTT 60
Malus           ATGGTGAAGGGTGTTGCTGTTCTCGGCTCCAGTGAGGGCGTTAAAGGAACCATCAGCTTT 60
Arachis         ------------------------------------------------------------
Brassica        ATGGGCAAGGGAGTGGCAGTCTTGAACAGCGGTGAGGGTGTTAAGGGGACTATCTTTTTC 60

Ipomoea         AGCCAAGAAGGAGATGGTCCAACCACAGTCACTGGAAACGTTTCGGGCCTCAAACCTGGT 120
Populus         ACCCAAGAAGGAGATGGCCCAACTACTGTAACTGGAAACCTTTCTGGTCTTAAGCCAGGC 120
Oryza           GTCCAAGAGGGAGATGGTCCCACCACTGTGACTGGAAGTGTCTCTGGCCTCAAGCCTGGT 120
Malus           GTCCAGGAGGGAGATGGCCCAACTACTGTGACTGGAAGTGTCTCTGGCCTCAAGCCTGGA 120
Arachis         ----AGGAAGGAAATGGTCCAACCACTGTGACTGGAAATCTTGCTGGCCTTAAGCCTGGT 56
Brassica        ACCCAGGAAGGAGACGGTGTGACCACTGTGACTGGAACAGTTTCTGGACTTAAACCTGGT 120
                    * ** *** * **    ** ** ** *******   *  * ** ** ** ** **

Ipomoea         CTTCATGGCTTCCATGTCCATGCCCTAGGTGACACAACAAATGGATGCATGTCTACTGGA 180
Populus         CTTCATGGCTTCCACGTCCATGCCCTTAGAGACACCACAAATGGCTGCATGTCAACTGGG 180
Oryza           CTCCATGGGTTCCATATTCATGCACTTGGTGACACCACCAATGGTTGCATCTCAACTGGG 180
Malus           CTTCATGGTTTCCATGTCCATGCTCTTGGAGACACAACAAACGGTTGCATGTCAACTGGG 180
Arachis         CTTCATGGGTTCCATGTCCATGCCCTTGGAGACACCACAAATGGTTGCCTGTCAACTGGA 116
Brassica        CTACATGGTTTCCATGTCCATGCTCTTGGTGACACCACTAACGGTTGCATGTCCACCGGT 180
                ** ***** *****  * ***** **  * ***** ** ** ** *** * ** ** **

Ipomoea         CCACATTTCAATCCTGCTGGAAAGGAGCATGGAGCTCCTGGAGACGATAACCGCCATGCC 240
Populus         CCGCATTTTAATCCTGTAGGCAAGGAGCATGGTGCCCCTGAGGATGAGAATCGTCATGCT 240
Oryza           CCACACTACAATCCTGCCGGAAAGGAGCATGGAGCACCAGAAGATGAGACCCGCCATGCT 240
Malus           CCACACTTCAATCCTGCTGGAAAAGAGCATGGTGCCCCTGAAGATGAGCTTCGCCATGCT 240
Arachis         CCGCATTTCAATCCTAACAACAAGGAGCATGGTGCCCCTGAAGATGAGAACCGCCATGCT 176
Brassica        CCACATTTCAACCCTGATGGTAAACAACACGGTGCCCCTGAGGATGCTAATCGTCATGCT 240
                ** ** *  ** ***      **  * ** ** ** ** *  ** *     ** *****

Ipomoea         GGTGATCTTGGAAACATCACGGTTGGAGAAGATGGTACTGCTT-CATTCACCATCACTGA 299
Populus         GGTGATCTGGGAAATGTCACTGTTGGTGATGATGGCACTGCTA-CTTTCACAATCATTGA 299
Oryza           GGTGATCTTGGAAATGTCACCGCTGGAGAAGATGGTGTTGCTAATATCCATG-TTGTTGA 299
Malus           GGCGATCTTGGAAACATCACTGCTGGGGACGATGGAACTGC-AACCTTCACGATTGTTGA 299
Arachis         GGTGATTTAGGAAATGTTAATGTTGGAGATGATGGAACTGTTAGC-TTCTCCATTTCCGA 235
Brassica        GGCGATCTAGGAAACATCATTGTTGGAGATGATGGAACTGCCA-CCTTCACAATCACTGA 299
                ** *** * *****  * *  * *** ** *****   **      * *    *    **

Ipomoea         CAAGCAGATTCCGCTTACTGGAGCAAATTCTGTTATTGGAAGAGCTGTTGTTGTTCATGG 359
Populus         CAAACAGATTCCTCTTACTGGACCACATTCCATTATTGGAAGGGCTGTTGTTGTTCATGG 359
Oryza           CAGTCAGATTCCACTTACTGGACCAAATTCAATCATTGGCAGAGCCGTCGTTGTGCATGC 359
Malus           CAAGCAGATTCCTCTCGCTGGACCACACTCTATCATTGGTAGGGCGGTTGTTGTCCACGC 359
Arachis         CAGTCAGATCCCTCTTAGTGGGCCAAACTCCATTGTTGGAAGGGCTGTTGTTGTCCATGC 295
Brassica        CTGCCAGATTCCTCTTAGTGGACCAAACTCTATTGTCGGAAGGGCTGTTGTTGTCCACGC 359
                *   ***** ** **   ***  ** * **  *  * ** ** ** ** ***** ** *

Ipomoea         TGATCCCGATGATCTTGGTAAAGGTGGCCATGAGCTCAGCAAAAGCACTGGAAATGCTGG 419
Populus         AGATCCTGATGATCTTGGCAAGGGAGGACATGAACTCAGCAAAACCACCGGTAATGCTGG 419
Oryza           CGATCCTGATGATCTTGGAAAGGGTGGGCACGAGCTGAGCAAGACCACCGGAAACGCTGG 419
Malus           AGACCCTGATGACCTTGGCAAGGGTGGACATGAGCTTAGCAAATCCACAGGAAATGCTGG 419
Arachis         TGATCCTGATGATCTTGGGAAAGGTGGGCATGAGCTTAGCAAATCCACTGGAAATGCTGG 355
Brassica        AGACCCTGATGACCTCNGAAAGGGAGGCCATGAACTCAGCTTGGCTACCGGAAATGCANG 419
                 ** ** ***** **  * ** ** ** ** ** ** ** ***      ** ** ** **  *

Ipomoea         CGGGAGGGTTGCCTGCGGTATCATTGGCCTGCAGGGTTAA 459
Populus         CGGCAGAGTAGCATGCGGTATTATTGGTCTGCAAGGTTGA 459
Oryza           TGGCCGTGTTGCTTGCGGGATCATCGGACTTCAAGGCTGA 459
Malus           TGGCAGGGTGGCTTGCGGTATTATTGGTCTGCAAGGATGA 459
Arachis         TGGCAGAGTAGCTTGTGGGATTATTGGTTTGCAAGGCTAG 395
Brassica        TGGCCGTGTTGCTTGTGGCATTATTGGTCTTCANGGCTAA 459
                 **  * ** ** ** ** ** ** **  * ** ** *
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Figure 1. Nucleotide sequence alignment of the cytosolic CuZn-SOD gene from different species using Clustal W. Representative plant species are *Ipomoea batatas* (JQ906095), *Populus suaveolens* (DQ481231), *Brassica juncea* (AF540558), *Oryza sativa* (L36320), *Arachis hypogaea* (DQ499511) and *Malus xiaojinensis* (AY646367). Degenerate primers (Table 1) for real time PCR were designed from sequences shaded in yellow.

liquid was carefully removed with a pipet. The RNA pellet was dissolved in 1 mL TE buffer and 0.9 volume of chilled isopropanol and 0.1 volume of 3.3 M sodium acetate were added, followed by precipitation at -80ºC for 1 h RNA pellets were collected by centrifugation in a microcentrifuge at 16,000×g at 4°C for 30 min, washed twice with 200 µl of 70% ethanol, and collected by centrifugation at 16,000×g at 4°C for 20 min. Pellets were dried at room temperature, and RNA was resuspended in 200 µl of autoclaved DEPC-treated water. The quality and quantity of the isolated RNA were verified by 1% formaldehyde denaturing agarose gel electrophoresis and spectrophotometry.

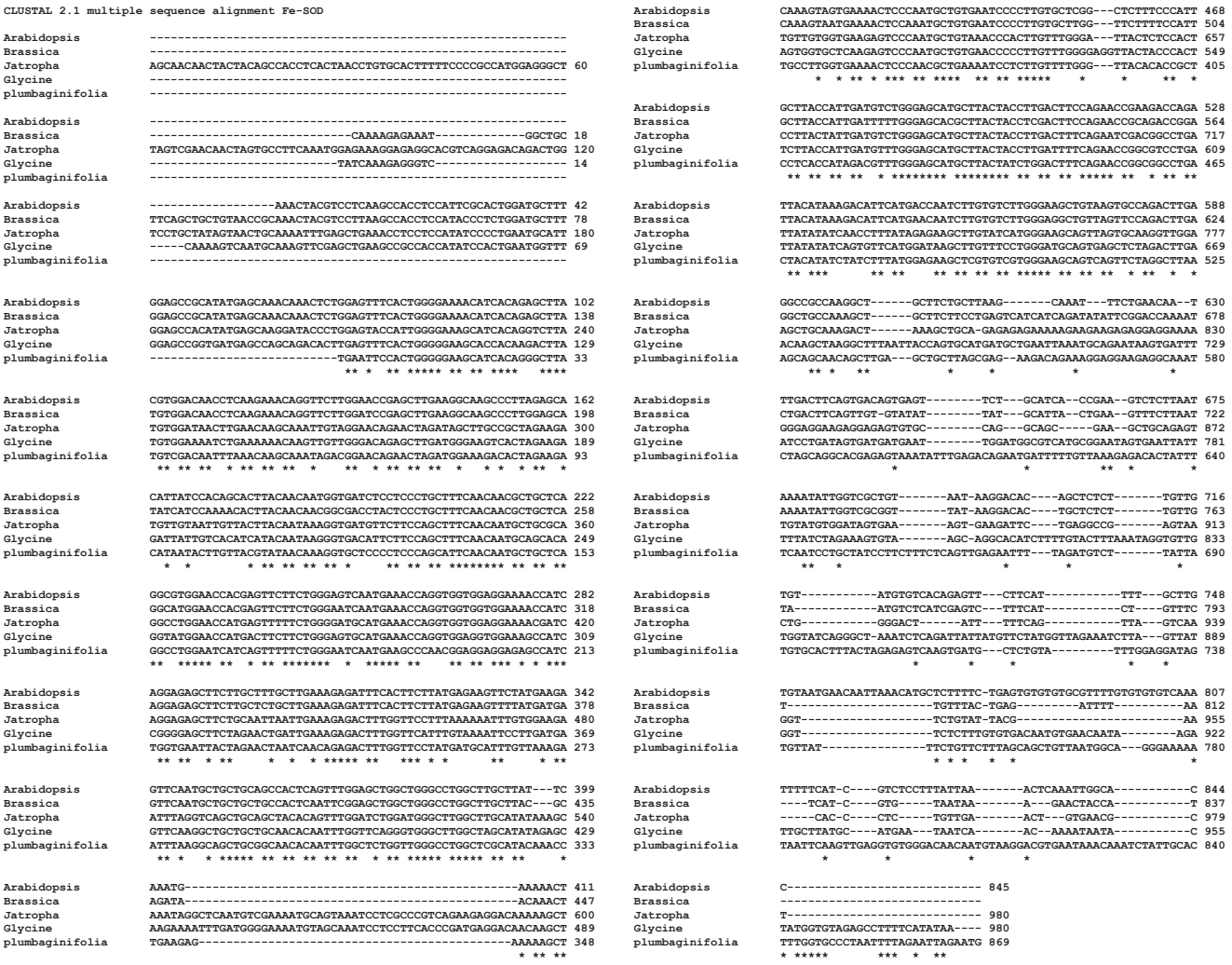

Figure 2. Nucleotide sequence alignment of the Fe-SOD gene from different species using Clustal W. Representative plant species are *Arabidopsis thaliana* (M55910.1), *Glycine max* (AAA33960.1), *Jatropha curcas* (JF509742.1), *Brassica oleracea* (JF720320.1), *Nicotiana plumbaginifolia* (M55909.1). Degenerate primers (Table 1) for real time PCR were designed from sequences shaded in yellow.

First-strand cDNA from 200 ng of total RNA was synthesized using AffinityScript QPCR cDNA Synthesis Kit following manufacturer's instruction using equimolar (10 pmole) concentration of oligo(dt) and random primer. In order to assess the integrity of cDNA prepared, Reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed taking 18s and SOD primers (Table 1).

Real-Time PCR analysis

Real-Time PCR reactions were performed in using QuantiTect SYBR Green PCR to detect dsDNA synthesis. Reactions were done according to kit instruction in 25 µl volumes containing 10 pmole of each primer and 50 ng of starting RNA. Three replications were done for each gene analysis of Real-time PCR. Dissociation curves for each amplicon were then analyzed to verify the specificity of each amplification reaction.

Relative gene expression data were analyzed using real-time quantitative PCR by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Expression levels (fold change) were determined as the number of amplification cycles needed to reach a fixed threshold in the exponential phase of the PCR reaction (CT). To assess the sensitivity and amplification efficiencies of the method three different template dilutions were checked. The amount of target were normalized to the housekeeping reference (18s rRNA) and used for $2^{-\Delta\Delta CT}$ calculation.

For relative quantification, expression of all the SOD isoform in root was taken as unity for fold change calculations.

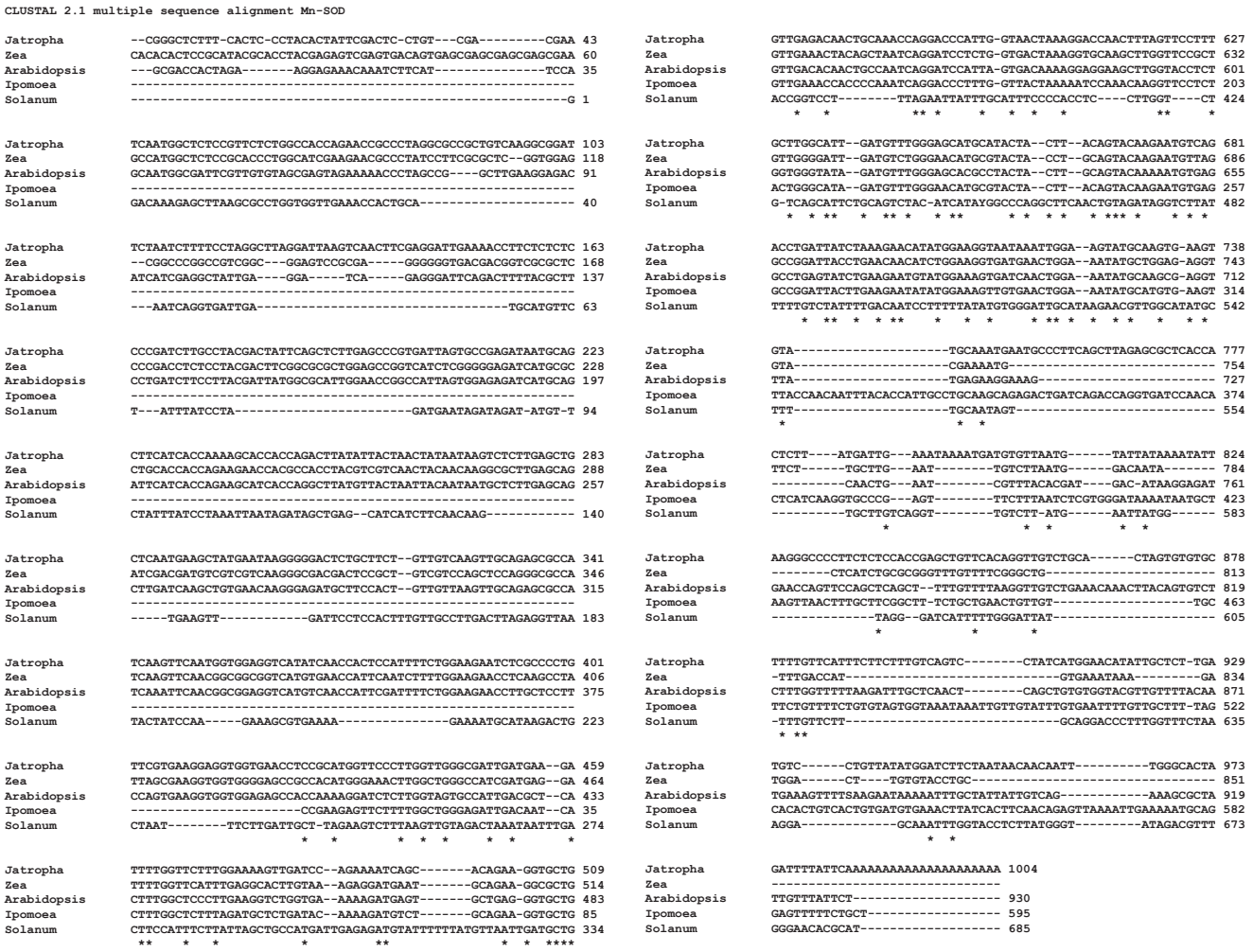

Figure 3. Nucleotide sequence alignment of the Mn-SOD gene from different species using Clustal W. Representative plant species are *Jatropha curcas* (JF509743.1), *Ipomoea batatas*(L36676.1), *Solanum bulbocastanum* (HQ856192.1), *Zea mays* (L19462.1), *Arabidopsis thaliana* (AY085319.1). Degenerate primers (Table 1) for real time PCR were designed from sequences shaded in yellow.

Results

SOD activity in crude extracts

A comparative analysis of total SOD activity in the crude extract of different organs of *I. carnea* is presented in Figure 4. SOD activity assays were performed with increasing protein concentration (Fig. 4A) and specific activity (units/ mg of protein) was calculated (Fig. 4B) in the linear range of increase in SOD activity with increase in protein concentration. SOD specific activity was maximum in case of apical and lateral bud, while root and petal show comparatively lesser activity. Leaf and stem showed moderate SOD activity as compared to other plant parts.

Higher SOD activities in the meristematic tissue like apical and lateral buds are obvious also. It is well documented that these fast dividing cells produces large amount of reac-

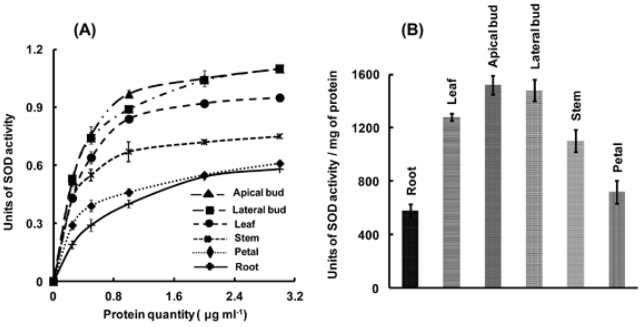

Figure 4. SOD activity in crude extracts of I.carnea. (A) as a function of increase in protein concentration (B) specific activity of the protein in terms of Units of SOD activity/ mg of protein.

tive oxygen species due to higher metabolic rate (Samis et al. 2002 ; Bi et al. 2011). Thus, increase in SOD activity in these tissues is physiologically essential in order to tolerate flux of ROS and hence prevent the plant from oxidative damage. Lesser SOD activity in non green tissues like root and petals could be argued for lack of photosynthesis machinery, the process which is accountable for largest production of superoxide is plant cells (Perl-Treves and Galun 1991; Murai and Murai 1996; Asada 1999). Therefore, these plants organs represent differential accumulation $O_2^{\cdot-}$ and plants are adapted to regulate the SOD activity based on local accumulation of $O_2^{\cdot-}$.

Purification and quality detection of total RNA

Electrophoresis of isolated RNA on 1% denaturating agarose gel stained with ETBR showed distinct 28S and 18S rRNA bands (Fig. 5A). Only those RNA samples with 260/280 ratio between 1.9 and 2.1 and 260/230 ratio greater than 2.0 were used for the cDNA preparation (Jain et al. 2006).

RT-PCR of the cDNA prepared using the housekeeping 18S rRNA and all three SOD isoform show a single distinct band in agarose gel (Fig. 5B-C) and hence proceeded for real time analysis.

Real time PCR analysis and quantification of relative gene expression

Presence of single sharp peak for both 18S rRNA and SOD as reflected in the dissociation curves (melting curves) ascertain the absence of primer dimers and non-specific amplification products. It also showed that the amplification has good reproducibility in each sample for all genes. However, both RT PCR and amplification curve (data not shown) of Fe-SOD shows slightly less efficiency, which might be due to higher degeneracy of the Fe-SOD sequence with the designed primer.

The quantitative expression cytosolic CuZn-SOD gene in different organs was evaluated by taking the amount of expression in root as unity (Fig. 6A). The relative expression level of the gene compared to root was highest in leaf (1.3 times), while expression was about 0.35-fold and 0.22-fold lower in case of apical and lateral bud respectively. The transcript abundance in stem and petals as compared to root was about 0.54 and 0.37.

Although, it was shown in earlier section that the total SOD activity was highest in buds, relative mRNA expression of the cytosolic CuZn-SOD gene was found to be least in these cases. These anomalous gene expression profiles suggest that distributions of different SOD isoforms in different organs of the plants are different. This is also supported by previous reports (Kliebenstein et al. 1998; Corpas et al. 2006) where it was found that the cytosolic CuZn-SOD plays a more important role in oxidative stress tolerance in roots as compared with the chloroplastic isoform. It is most likely

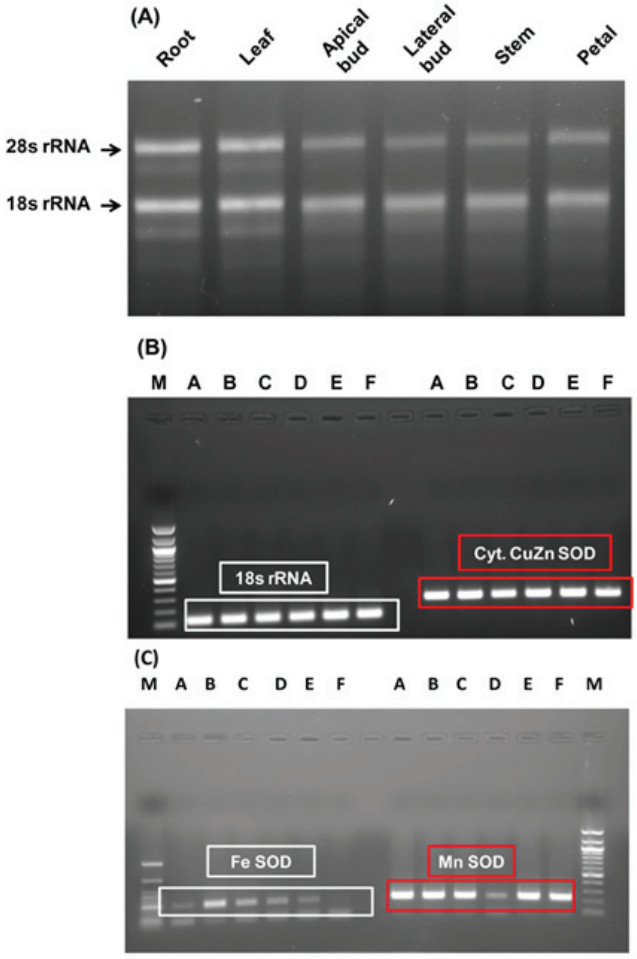

Figure 5. Purification of total RNA and reverse transcriptase PCR. (A) Resolution of total RNA on formaldehyde agarose gel. (B) PCR of the corresponding cDNA using 18s rRNA and CuZn-SOD primer and (C) Fe-SOD and Mn-SOD. Sequence of representative cDNA from A-F is root, leaf, apical bud, lateral bud, stem and petal.

that, others isoforms of SOD like chloroplastic CuZn-SOD, Fe-SOD and Mn-SOD might be contributing to the higher activity of the buds.

Evaluation of transcript level of Fe and Mn-SOD isoforms also advocate this fact. The relative expression level of the Fe-SOD was highest in leaf (6.3 times), followed by apical bud and lateral bud while expression was comparable in case of stem and petal (Fig. 6B). Variation in Fe-SOD transcript abundance in these organs primarily represent the presence of chloroplast and those organs where chloroplast is absent shows lower level of expression, which is primarily basal level only.

The expression level of Mn-SOD gene was highest in apical bud followed by lateral bud while transcript abundance of the gene was average in root, leaf, stem and petal (Fig. 6C). These observations suggest that both apical and lateral bud

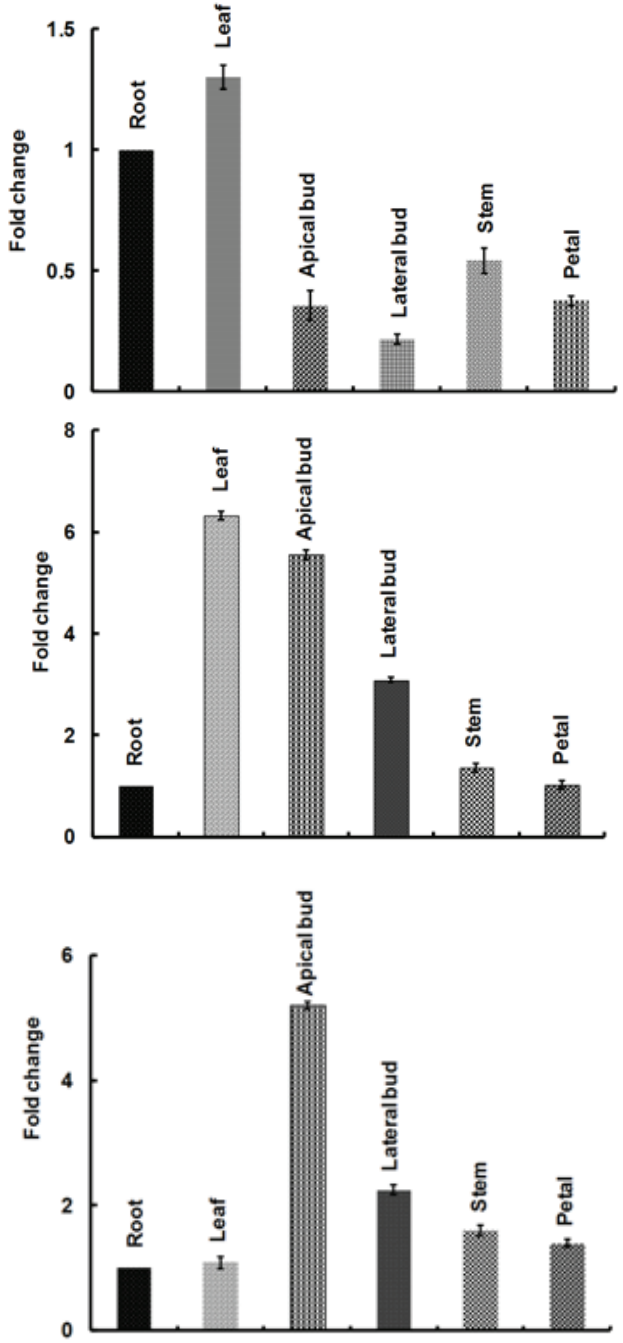

Figure 6. Relative quantification of SOD expressions in different organs of *I.carnea*. Quantification of gene expression was evaluated by taking the amount of expression in root as unity and expressed in terms of fold change. (A) CuZn-SOD (B) Fe-SOD (C) Mn-SOD.

have higher accumulation $O_2^{\cdot-}$ which might be due to high rate of metabolism and since Mn-SOD is the major isoform present in mitochondria, so higher expression of Mn-SOD in these meristematic organs strongly advocate its higher abundance to prevent plant from oxidative injury. So, perhaps Mn-SOD contributes towards higher SOD activity of buds as shown in earlier section.

Discussion

Reactive oxygen species (ROS) are produced as a normal product of plant cellular metabolism. ROS are always formed by the inevitable leakage of electrons onto O_2 from the electron transport activities of chloroplasts, mitochondria, and plasma membranes or as a by product of various metabolic pathways operating in different cellular compartments (Bannister et al. 1987a). Various Environmental stresses such as drought, salinity, chilling, metal toxicity, and UV-B radiation as well as pathogens attack can lead to enhanced production of ROS within plant tissues due to disruption of cellular homeostasis (Bowler et al. 1994). Scavenging of excess ROS is achieved by highly efficient antioxidative machinery comprising of both nonenzymatic and enzymatic antioxidants. The enzymatic components include superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), enzymes of ascorbate-glutahione (AsA-GSH) pathway such as ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR). Ascorbate (AsA), glutathione (GSH), carotenoids, tocopherols, and phenolics serve as potential nonenzymatic antioxidants within the plant cell (Noctor and Foyer 1998). However, plants rely upon the unique enzyme superoxide dismutase (SOD) to detoxify superoxide. That's why SODs are ubiquitous enzymes present in all phyla and various isoforms of this class of enzymes are distributed in different cellular compartments. The presence of multiple SOD isoforms raises the possibility that each protein may protect plants against a subset of oxidative stresses and that a variety of SODs are deployed to fully combat environmental stresses (Bowler et al. 1994; Alscher et al. 2002).

There have been several studies showing the importance of SODs in combating environment stresses by developing plants overexpressing different isoforms of the enzyme. However, there are some disparities among transgenic plants overexpressing SOD (Tepperman and Dunsmuir 1990; Gupta et al. 1993). It is also essential to obtain deeper insights into the relationship between cellular localization and specific function of each SOD isoforms. However, tissue and organ specific expression of SOD activity and relative expression of different isoforms are still lacking in literature.

In some cases, tissue specific expression were analysed by fusing the 5' upstream regulatory region of these genes to the beta-glucuronidase reporter gene and differential tissue specificity were checked in transgenic plants. Those studies were confined only to one gene at a time (Van Camp et al. 1996). Therefore, in this study we report the total SOD activity in different organs of *Ipomoea carnea* and further extended to analysis of transcript abundance of cytosolic CuZn-SOD,

Fe-SOD and Mn-SOD by real time PCR. Our observations demonstrate that expression of different isoforms of SODs are developmentally regulated and hence provide a valuable clue about the existence of a specific isoform of SODs in particular organ of the plant.

The comparison of total SOD activity in the crude extract of different organs of *I. carnea* showed clear discrepancies. Our observation reveals that activity was maximum in case of apical and lateral bud. These buds of plant represent the highly meristematic region. Hence, it is apparent that the high metabolic rate might result in higher production of ROS; an event which might be responsible for higher SOD activity. However, Root and petal shows least activity which might be due to lack of photosynthesis and/or lesser metabolic rate in these organs. These data emphasize the critical role of subcellular superoxide dismutase location and strongly advocate that enzymatic activity of SOD is differentially regulated at different organs of the plant depending upon its developmental and physiological conditions.

CuZn SOD is the most abundant SOD isoenzyme in many plant species (Bowler et al. 1994; Alscher et al. 2002). In this report, we have analysed the relative expression of major isoform of CuZn-SOD which is localised in cytosol. Our observations reveal that expression of cytosolic CuZn-SOD is slightly higher in leaf while other organs show less abundance of the gene. This also supports previous reports which suggest that cytosolic CuZn-SOD plays major role in scavenging $O_2^{\cdot-}$ (Perl-Treves and Galun 1991; Murai and Murai 1996) and hence this isoforms is uniformly distributed in different tissues of the plant.

On the other hand, expression level of Fe-SOD was found to be highest in leaf followed by apical bud and lateral bud, while expression was least non photosynthetic organs like root, stem and petal. To date most of the Fe-SODs found are chloroplastic, so abundance of chloroplast in organs probably determine abundance of Fe-SOD mRNA abundance in these organs (Bowler et al. 1994; Okamoto and Colepicolo 1998; Alscher et al. 2002). In addition to Fe-SOD, CuZn-SOD is also present in chloroplast and both of these enzymes are responsible for the efficient removal of the superoxide formed during photosynthetic electron transport and hence function in reactive oxygen species metabolism. The availability of copper is believed to be a major determinant of CuZn-SOD and Fe-SOD expression (Pilon et al. 2011). However, in our case since all the plant organs belongs to same condition (copper present in the soil), expression pattern of Fe-SOD/ CuZn-SOD represent the true value and copper is not a detrimental factor here. Rather, presence/ absence of chloroplast are the sole determinant of the gene expression.

Mn-SODs are found in mitochondria; with only exceptions are watermelon and pea, where it is found in peroxisomes also (del Río et al. 2003; Rodríguez-Serrano et al. 2007). Decrease in Mn-SOD may leads to reduced root growth and affects Tricarboxylic Acid Cycle (TCA) Flux and mitochondrial redox homeostasis (Morgan et al. 2008). Thus, regulation of Mn-SOD is critical in those tissue where generation $O_2^{\cdot-}$ from mitochondria is very high. The transcript abundance of Mn-SOD gene in apical bud and lateral bud suggests higher accumulation $O_2^{\cdot-}$ in these meristematic tissues which might be due to high rate of metabolism and Mn-SOD play critical role here to prevent plant from oxidative injury (Seguí-Simarro et al. 2008).

In conclusion, expression patterns of SOD isoenzymes give insights into their probable functions in different tissues and development stages. The SODs in different compartments must be differently regulated at the level of gene expression by site-specific oxidative stress. Our findings demonstrate that distinct regulation mechanisms might be involved in the expression of SODs in different organs of *Ipomoea carnea*. All these cases provide evidence of the heterogeneous distribution of SOD isozymes in higher plant species, and suggest that each SOD isoenzyme must have a specific function probably related to its cellular and subcellular localization.

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