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Full Length Research Paper

Molecular cloning and characterization of a new peroxidase gene (*OvRCI*) from *Orychophragmus violaceus*

Dai Qilin[1,2], Wang Jin[2,3], Feng Bin[3], Liu Tingting[3], Chen Chen[4], Lin Honghui[1*] and Du Shizhang[2*]

[1]Key Laboratory of Bio-resources and Eco-environment (Ministry of Education), College of Life Sciences, Sichuan University, Chengdu 610064, China.
[2]Plant Genomics Research center, Mianyang Normal College, Mianyang 621000, China.
[3]Plant Biotechnology Research Centre, Southwest University of Science and Technology, Mianyang 621000, China.
[4]School of Pharmacy, Fudan University, Shanghai 200030, China.

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A new peroxidase gene from *Orychophragmus violaceus* was cloned. The full-length cDNA of *O. violaceus* peroxidase gene *(OvRCI*, GenBank. Acc. No. AY428037) was 1220 bp and contained an 1128 bp open reading frame encoding a protein of 375 amino acids. Homology analysis and molecular modeling revealed that *OvRCI* strongly resembled other peroxidase genes. Quantitative real-time PCR analysis revealed that it was a constitutively salt-inducible gene and its transcript level was most abundant after 24 h treatment with 200 mmol.L^{-1} sodium chloride. Our studies suggested that *OvRCI* was a new member of the family of recently cloned peroxidase genes.

Key words: *Orychophragmus violaceus*, OvRCI, quantitative real-time PCR.

INTRODUCTION

Salt stress is a major factor limiting crop production because it affects almost all plant functions (Bohnert and Jenson, 1996). In saline environment, membrane disorganization, reactive oxygen species (ROS, e.g. O_2^- and H_2O_2) increase, metabolic toxicity, inhibition of photosynthesis and regulation of saline metabolism attenuated nutrient acquisition are factors that initiate more catastrophic events (Hasegawa et al., 2000). Salinity mainly causes both hyper-osmotic stress and hyper-ionic toxic effects and the consequence can be plant demise (Hasegawa et al., 2000). Osmotic stress mainly brings about a water deficit that results from the relatively high solute concentrations in osmotic stress (Zhu, 2002).

An increasing evidence also suggests that high salinity induces oxidative stress (Gosset et al., 1996; Góemez et al., 1999; Savouré et al., 1999), which is a key underlying component of most abiotic stresses (Mittle, 2002; Apel and Hirt, 2004) and a major limiting factor of plant growth in the field (Mittler, 2006). Metabolic change is a key part of the response to oxidative stress in microbes and higher plants (Charles et al., 2007). The metabolism of chloroplasts and mitochondria under NaCl stress favored the formation of O_2^- and H_2O_2 in two pea cultivars differing in sensitivity (Hernandez et al., 1993, 1995). ROS are generated as by-products of plant cellular metabolism (Kavitha et al., 2008) and also important as signaling molecules (Mittler, 2002). Elevated production of ROS can seriously disrupt cellular homeostasis and normal metabolisms through oxidative damage to lipids, protein and nucleic acid (Meloni et al., 2003), which leads to cellular dysfunction and ultimately cell death (Halliwell, 2006).

To avoid these cell death scenarios, plants invoke a molecular response that allows them to cope with and adapt to the oxidative stress situation (Charles et al., 2007). Plant cells can synthesize organic compounds, some soluble small-molecular-weight osmolytes which prevent from water efflux and maintain cellular expansion to keep osmotic balance and eliminate ROS to increase plant cells salt-tolerance under salt conditions by metabolic

*Corresponding author. E-mail: wjdsz@vip.sina.com.cn; honghuilin@vip.sina.com.

change. Frequently, the organic compounds include betaine (glycine betaine, praline betaine, β-alanine betaine, vitriol and dimethyl sulfonium propionate, DS MP), sugars (mainly sucrose and fructose), sugar alcohol (glycerol, ononitol, ectoine and methylated inositols), complex sugars (trehalose, rafinose, fructans), mannitol and amino acid (proline) (Paul et al., 2000; Bohnert and Shen, 1999; Hare and Cress, 1997; Jain and Selvaraj, 1997; Nelson et al., 1998).

Therefore, antioxidant resistance mechanisms may provide this strategy to entrance salt tolerance (Hernandez et al., 2001). Moreover, plants have evolved efficient antioxidant systems that can protect them from the damaging effects of oxidative stress (Asada, 1999). These mechanisms employ ROS scavenging enzymes which detoxify plant by scanvenging oxygenic radical (Roxas et al., 1997; Allen et al., 1997). Peroxidase is one of such small molecular antioxidant enzymes, which catalyzes the conversion of H_2O_2 to H_2O and O_2 (Asada, 1999) and plays a key role in defense of plant against biotic and abiotic stress (Mark et al., 1987).

Orychophragmus violaceus (*O. violaceus*) belongs to *Orychophragmus*, *Brassica* in Cruciferae, mainly distributing in China. It has been found that *O. violaceus* subjected to salt stress of 150 mmol.L^{-1} NaCl solutes displays strong tolerance and normally grows, flowers and seeds through planting in field, but *Arabidopsis* belongs to Cruciferae only grows in 70 mmol.L^{-1} NaCl solutes. Up to now, there has been no report on the molecular cloning of peroxidase gene from *O. violaceus*. In the present study, we report on the screened peroxidase gene from the cDNA Library of *O. violaceus* using the *Arabidopsis* peroxidase gene (*RCI*) as the probe and then *O. violaceus* peroxidase gene (*RCI*) sequence is obtained by sequencing and homology analysis. The expression pattern of *O. violaceus* peroxidase gene (*RCI*) under different NaCl concentrations is also investigated.

MATERIALS AND METHODS

Plant material and growth conditions

O. violaceus seeds (provided by Genetic Lab in Sichuan University) were sterilized in 5% sodium hypchlorite solution for 10 min followed by 3 rinses with sterile distilled water and then sown in plastic containers (40 x 30 x 10 mm). The seedlings were irrigated on alternative days with full-strength Hoagland nutrient solution. Twenty-three days later, *O. violaceus* were divided into two groups (A and B group). Various concentration (0, 100, 150, 200, 250 and 300 mmol.L^{-1}) NaCl were added into the nutrient solution of A group. *O. violaceus* of B group with 200 mmol.L^{-1} NaCl solute was treated for 0, 6, 12, 24 and 48 h.

cDNA Library construction and *OvRCI* gene cloning

Young leaves from the plant treated with 200 mmol.L^{-1} NaCl for 24 h were powdered in liquid nitrogen with mortar and pestle. Total RNA (free DNA) was extracted according to the handbook of nucleic acid isolation and purification (Cat. No.W6674, Watson, China). The cDNA Library was performed according to the SMART™ cDNA Library kit (Cat.No.634901 Clontech, USA). All of the cDNA Library clones were screened on the LB medium at 37℃ using the probe with 300 bp designed according to peroxidase gene sequence (gi:30693142) from *Arabidopsis*. Six positive clone were screened out on LB plates. The inserts of these plasmids isolated from the *Escherichia coli* cells were sequenced in Shanghai Sangon Biotech Company. The sequences of these contigs were assembled together and the sequence was determined.

Above these fragments were assembled into a full-length cDNA (*OvRCI*) through alignment and deletion of the overlapping fragment with VNTI 6.0 software. Based on the full-length cDNA sequence, gene specific primers *OvRCI* F1 (5-ATGCATTTCTCT TCGTC TTC AACAT-3) and *OvRCI* R1 (5-TTATTCATTAATATATA TATGTATTCTTG-3) were used for the amplification of coding region fragment of *OvRCI* by RT-PCR following the manual described by the manufacturer (TAKARA RT-PCR kit) and the amplified coding sequence of *OvRCI* was inserted into pGEM-T vector (Promega) followed by sequencing.

Analysis of expression of *OvRCI* under salt stress

The seeds of *O. violaceus* were sterilized in 5% sodium hypochlorite solution for 10 min followed by 3 rinses with sterile distilled water and then sown randomly in thoroughly washed sand in plastic containers (40 × 30 × 6 cm^3) with drainage holes in the bottom. The seedlings were irrigated on alternative days with full-strength Hoagland nutrient solution. After 8 d, the seedlings were transplanted into bigger plastic pots (22.5 cm in diameter and 22.5 cm in depth) and continued irrigating with the same fresh nutrient solution. Twenty-three days later, the seedlings were divide into A and B groups. *O. violaceus* in A group were treated by various concentration (0, 100, 150, 200, 250 and 300 mmol.L^{-1}) NaCl solution. *O. violaceus* in B group were treated by 200 mmol.L^{-1} NaCl solution at various durations (0, 6, 12, 24 and 48 h).

Total RNA was extracted from young leaves of the plants in A and B groups using TRIzol reagent (GIBCO-BRL). RNase-free DNaseI (Takara, Japan) was used to digest the genomic DNA in the total RNA preparation. One hundred microgram of total RNA was then synthesized into 1st strand cDNA using a Reverse-Transcription Kit (Takara, Japan). The expression levels of the *OvRCI* gene were determined using the iCycler iQ Real-time PCR Detection System (Bio-Rad) according to the manual of Two-step Quanti Tect SYBR Green PCR Kit. Data processing was done by iCycler real-time detection system software (version 2.0). The primers used for qRT-PCR amplifications were designed with Primer 12 Express 2.0 (Applied Biosystems) for PCR products that were 100 ~ 150 bp in length. Expression levels were normalized using values obtained for the housekeeping gene β-actin. All real-time PCRs were performed in duplicate in three independent experiments.

Measurement of OvRCI activity

After subjected to salt stress, the soluble extracts were extracted from *O. violaceus* young leaves of above A and B groups grinded with mortar and pestle under 0℃ condition. OvRCI activity was determined spectrophotometally by the method of Nishikawa et. al., (2003). The assay mixture contained 50 mmol.L^{-1} PBS (pH7.0), 1mmol.L^{-1} EDTA, 0.5 mmol.L^{-1}M L-ascorbic acid and the soluble extracts in plant leaves. The reaction was initiated with the addition of 0.1 mmol.L^{-1} H_2O_2. Enzyme activity was determined by measuring the decrease in absorbance of ascorbate at 290 nm over 3 min at 25℃.

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                                                        at ct t
    ct cggt aaacagaat caact agt t t t gt t t t t cct ct t t caaaa
  51 at gcat t t ct ct t cgt ct t caacat cgt ccact t ggacaat ct t a
     M  H  F  S  S  S  S  T  S  S  T  W T  I  L
  96 at cacat t gggat gt ct t at gct t cat gcat ct t t gt ccgct gct
     I  T  L  G  C  L  M  L  H  A  S  L  S  A  A
 141 caact cacccct acct t ct acgat aggt cat gt cct aat gt cact
     Q  L  T  P  T  F  Y  D  R  S  C  P  N  V  T
 186 aacat cgt acgagaaaccat t gt aaat gagt t aaggt cggaccct
     N  I  V  R  E  T  I  V  N  E  L  R  S  D  P
 231 cgt at cgct gcgagcat cct t cgt ct t cact t ccacgact gct t t
     R  I  A  A  S  I  L  R  L  H  F  H  D  C  F
 276 gt t aat ggt t gt gacgcat ccat ct t gt t agacaacacgacat ca
     V  N  G  C  D  A  S  I  L  L  D  N  T  T  S
 321 t t t cgaacagagaaagat gcgt t t ggaaacgcaaat t cggct cgg
     F  R  T  E  K  D  A  F  G  N  A  N  S  A  R
 366 ggat t t ccagt gat t gat agaat gaaagct gct gt gacgcat cga
     G  F  P  V  I  D  R  M  K  A  A  V  T  H  R
 411 t ct t gt t acacaacacgacat cr t t ccgaacagagaaagat gcaa
     S  C  Y  T  T  R  H  X  S  E  Q  R  K  M  Q
 456 t yggaaacgcaaact cggct agr ggat t t cckgt gct t gat acaa
     X  E  T  Q  T  R  L  X  D  F  X  C  L  I  Q
 501 t gcaagccgcagt ggagagggct t gcccr agaaccgt t t cat gcg
     C  K  P  Q  W  R  G  L  A  X  E  P  F  H  A
 546 cagat at gct caccat cgct gct caacaat ct gt cacgt t ggcag
     Q  I  C  S  P  S  L  L  N  N  L  S  R  W  Q
 591 gaggt cct t ct t ggagr gt t cct t t ggggagaagagacagyt t ac
     E  V  L  L  G  X  F  L  W  G  E  E  T  X  Y
 636 aagcat t t t t yr at ct akct aat ggt t aat ct t ccwt ct ccat t c
     K  H  F  X  I  X  L  M  V  N  L  P  S  P  F
 681 kycacact t ccagaact t aaagacagat t t agaaat gt t ggcct m
     X  T  L  P  E  L  K  D  R  F  R  N  V  G  L
 726 gaccgt cct t ct gat ct ygt t gct ct at ccgggcccamcmt aawr
     D  R  P  S  D  L  V  A  L  S  G  P  X  X  X
 771 r t aaact at gcccccct t aat ggcaat cgaagt gcct t ggt acat t
     X  N  Y  A  P  L  M  A  I  E  V  P  W  Y  I
 816 t t gat ct acgt acgcct acggt t t t cgacaacaaat act acgt gc
     L  I  Y  V  R  L  R  F  S  T  T  N  T  T  C
 861 at ct caaagagcgaaaaggt ct t at ccagagcgaccaagagt t gt
     I  S  K  S  E  K  V  L  S  R  A  T  K  S  C
 906 t ct ct agccccaat gccact gacacaat cccct t ggt gcgagcat
     S  L  A  P  M  P  L  T  Q  S  P  W  C  E  H
 951 at gct gat ggcacacaaacat t ct t caat gcat t t gt ggaggcaa
     M  L  M  A  H  K  H  S  S  M  H  L  W  R  Q
 996 t gcat aggat gggaaacat t acaccaact acaggaact caaggac
     C  I  G  W  E  T  L  H  Q  L  Q  E  L  K  D
1041 aaat cagat t gcact gt agagt t gt gcact ccaact ct ct gct cc
     K  S  D  C  T  V  E  L  C  T  P  T  L  C  S
1086 at gat gt ggt ggat at cgt t gact t t gt t agct ct at gt gagaat
     M  M  W  W  I  S  L  T  L  L  A  L  C  E  N
1131 t gt t t acccaat at gt ggct acaagaat acat at at at t aat gaa
     C  L  P  N  M  W  L  Q  E  Y  I  Y  I  N  E
1176 t aa aact ct caagacgt t t act t gagaacaaaaaaaaaaaaaaaa
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Figure 1. The full-length cDNA sequence and deduced amino sequence of *OvRCI* gene. The start codon (atg) is underlined and the stop codon (taa) is underlined and in italics.

RESULTS AND DISCUSSION

Molecular cloning and characterization of *OvRCI*

After cDNA library construction, determining the titer (e.g., pfu/µl) of the unamplified library would give us an estimate of the number of independent phage and independent clones in the library. A library having at least 1×10^6 independent clones, in most cases, was representative of the mRNA complexity. In this study, cDNA diluted positive is 1.65×10^6 and its recombinant rate reached over 90%.

Twelve primarily-screened 150 mmol.L^{-1} Petri dishes were plated, from which twenty-six positive cDNA clones identified with isotope-labeled oligo-nucleotide probe were picked out and preserved in SM buffer. The twenty-six clones were plated on 90 mmol.L^{-1} Petri dishes again, from which eleven positive clones were picked out. Six of the eleven clones contained the cDNA insert fragment and the six cDNA inserts were isolated and sequenced.

After six positive clones were sequenced, the cDNA sequence analysis was assembled into a CONTIG by DNASTAR software and get a full-length cDNA of *OvRI.* Using the method mentioned in the paper, the full-length cDNA sequence of *OvRCI* (GenBank Acc. No. AY428037) was obtained through RT-PCR reaction. The full-length cDNA of *OvRCI* was 1220 bp with an open-reading frame of 1128 bp, a 5-untranslated region of 50 bp and a 3-untranslated region of 42 bp. The amino acid sequence deduced from the open-reading frame revealed that the *OvRCI* encoded a protein of 375 amino acids with a calculated molecular weight of 44392.79Da. These predictions were made based on a web analysis program/on web analysis software (http://us.expasy.org). The complete nucleotide sequence and the deduced amino acid sequence of *OvRCI* are shown in Figure 1.

The deduced OvRCI protein was used in multiple alignment analysis using the ClustalX program; the results showed that, at the amino acid level, OvRCI had 64 and 59% identities respective with the peroxidase from *Arabidopsis thaliana* and *Brassica napus*. The putative peroxidase domain of OvRCI resided in the N-terminal region of the protein, while the C-terminal putative regulatory domain of OvRCI showed low sequence homology with that of AtRCI.

Analysis of expression of OvRCI under salt stress

Real-time PCR analysis was carried out to establish the expression profile of *OvRCI* of *O. violaceus* plants under salt stress. The results showed that, *OvRCI* expressed at different level under the various concentration NaCl (0, 100, 150, 200, 250, 300 mmol.L^{-1}) for 24 h. There were a little express of *OvRCI* without salt treatment, while its expression increased under 100 mmol.L^{-1}NaCl, 150 mmol.L^{-1}NaCl salt treatments and reached its highest level under 200 mmol.L^{-1}NaCl and then began to decrease

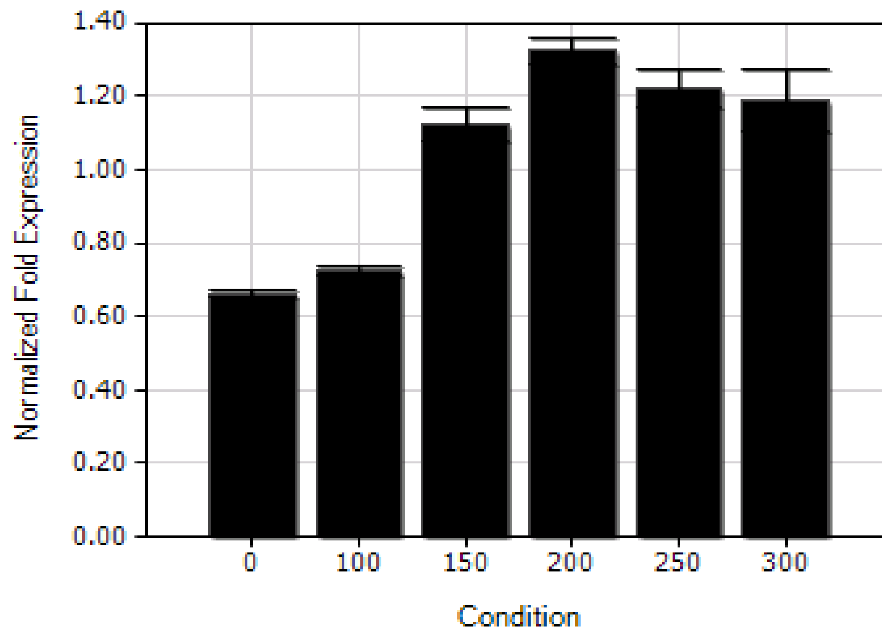

Figure 2. The expression profile of *OvRCI* gene under the various NaCl concentration (0, 100, 150, 200, 250 and 300 mmol.L^{-1}) for 24 h determined by quantitative RT-PCR (actin gene as internal standard). Fold inductions are the ratios of the copies of *OvRCI* genes and actin in *O. violaceus*.

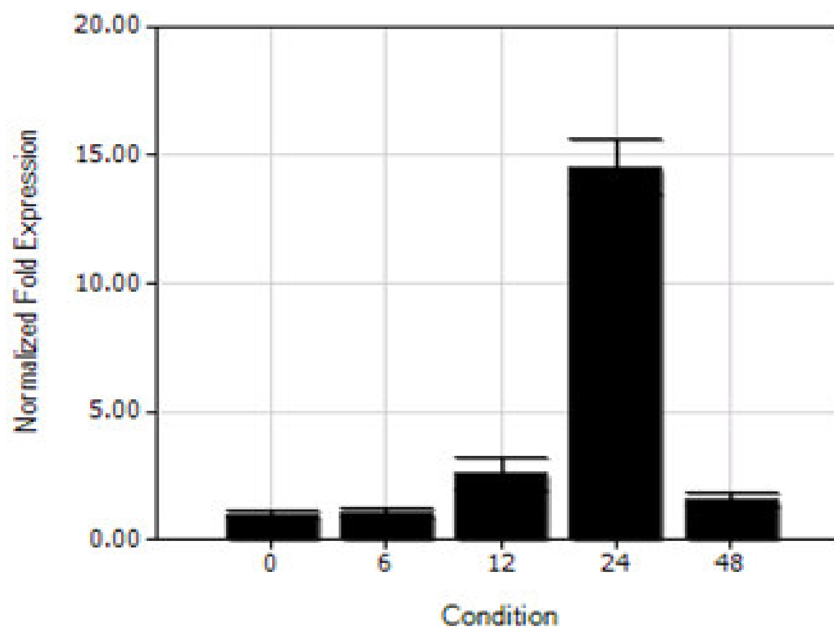

Figure 3. The expression profile *OvRCI* gene under 200 mmol.L^{-1} NaCl for 0, 6, 12, 24 and 48 h determined by quantitative RT-PCR (actin gene as internal standard). Fold inductions are the ratios of the copies of *OvRCI* genes and actin in *O. violaceus*.

(Figure 2). On the other hand, we exposed *O. violaceus* plants to 200 mmol.L^{-1}NaCl for 0, 6, 12, 24 and 48 h. The *OvRCI* gene expressed at a low level without salt treatment, while its expression increased after the salt treatments and reached its highest level after 24 h salt treatment (Figure 3). This result indicated that the

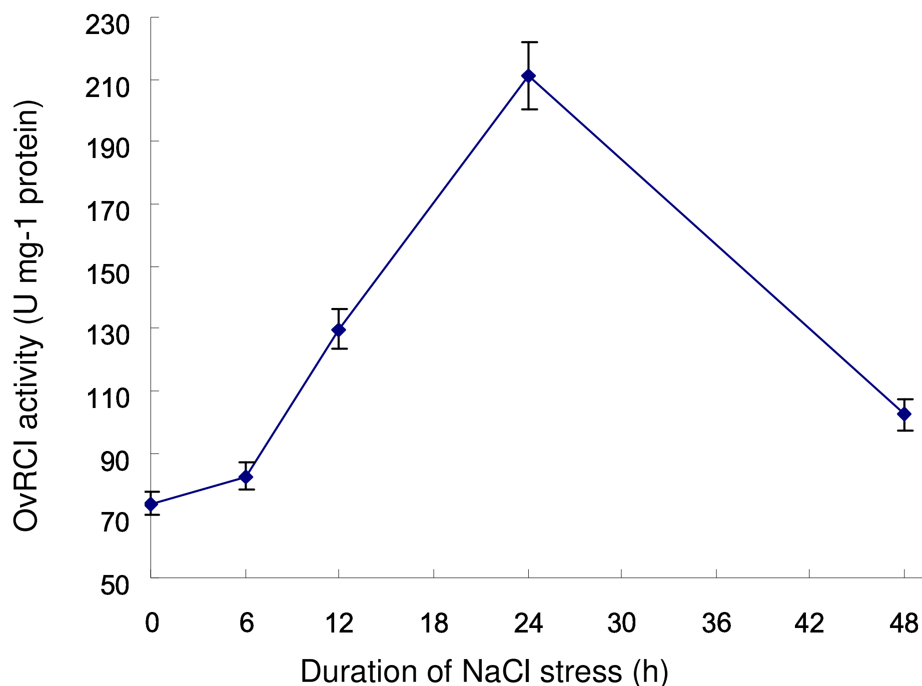

Figure 4. Changes of OvRCI activity in *O. violaceus* under 200 mmol.L^{-1} for 0, 6, 12, 24 and 48 h. The data represent the means and standard errors of three replications.

expression of *OvRCI* was salt-inducible

Changes of RCI activity under various salt stress conditions

After subjected to salt stress, the soluble extracts in plant leaves were completed under 0℃ condition. The effect of salt stress on total RCI activity was examined in leaves of *O. violaceus*. The result showed that there was a gradual increase in total RCI activity from 0 to 24 h, with the lowest OvRCI activity at 0 h, a little increasing at 6 h, distinct increasing at 12 h, peaking at 24 h and declining at 48 h. Compared to unstressed control (0 h), the RCI activity of *O. violaceus* leaves at 24 h had an higher increase of 76% (Figure 4). In the same time, after subjected to salt stress of various concentration salt solutes, the effect of salt stress on total RCI activity in leaves of *O. violaceus* were examined (Figure 5). A gradual increase in the total RCI activity was observed from 0 - 300 mmol.L^{-1}, with a little activity of OvRCI with 0 mmol.L^{-1}, obviously increasing under 100 mmol.L^{-1}, peaking under 200 mmol.L^{-1} salt stress and declining under 250 and 300 mmol.L^{-1}.

Ascorbate peroxidase (APX) activity was found to increase along with activities of other anti-oxidant enzymes like catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GSH reductase) in response to various environmental stress factors, suggesting that the components of ROS-scavenging systems were co-regulated (Shigeoka et al., 2002). In *Bruguiera parviflora*, a mangrove species, scorbate-mediated H_2O_2 degradation was enhanced 2.5 fold when plants were exposed to salt stress in the first week and 4.5 fold at 45 days of salt exposure (Parida, et al., 2004). The high levels of transcripts encoding this APX enzyme may reflect a particularly great need to prevent mitochondrial membrane damage in mangrove tissues (Nguyen et al., 2008). All members of the APX gene family, irrespective of the isoforms they encode, were ultimately associated with general cellular metabolism, stress response, signaling processes or the development of chloroplasts (Kavitha et al., 2008).

Conclusion

Sequence analysis suggested that the *OvRCI* may belong to peroxidase family. The effect of salt-mediated oxidative stress on transcript levels of *OvRCI* was analyzed in this study. Salt stress imposed oxidative stress transiently up-regulated *OvRCI* levels in *O. violaceus*. The inducibility of *OvRCI* by salt stresses was a characteristic feature, indicating its roles in the detoxification of metabolites generated by oxidative stresses. The cDNA sequences, predicted ORF and real-time PCR analyses in transcript levels of *O. violaceus* genes related to ROS scavenging were provocative for our understanding of oxidative stress responses in *O. violaceus*. The data above suggests that oxidative stress induced

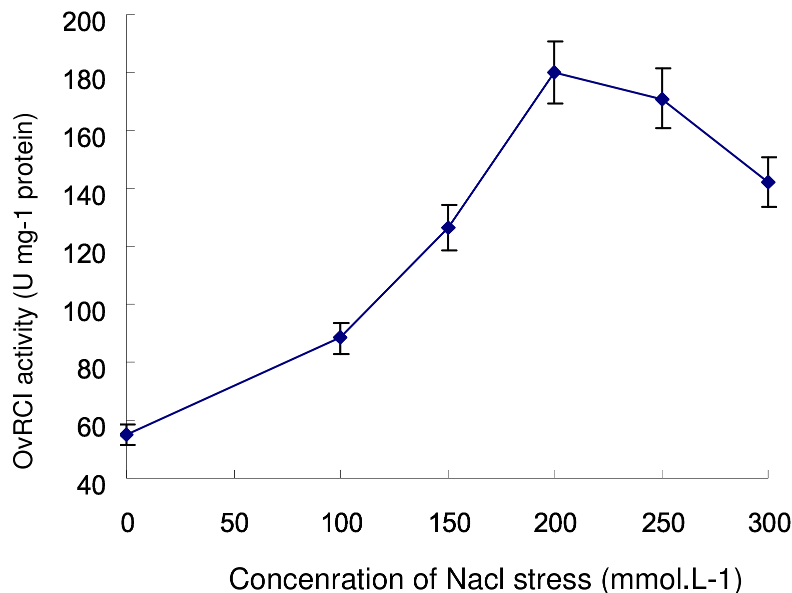

Figure 5. Changes of OvRCI activity in *O. violaceus* under 0, 100, 150, 200, 250 and 300 mmol.L^{-1} for 24 h. The data represent the means and standard errors of three replications.

increase in the transcript of *OvRCI* and increase in total OvRCI activity in leaves of *O. violaceus* might have a role in mitigating the deleterious effect of oxidative stress brought about by salt. Therefore, RCI may play an important role in removing H_2O_2 and protecting cells from oxidative damage.

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