BIOLOGIA PLANTARUM 59 (3): 456-462, 2015 DOI: 10.1007/s10535-015-0524-z

A germin-like protein gene of rice increased superoxide dismutase activity in transformed tobacco

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Abstract

Germin and germin-like proteins (GLPs) are a broad and diverse family of developmentally regulated proteins widely distributed in plants. *Oryza sativa* L. harbours a large family of GLPs and serves as a good model for their study. In the present study, a germin-like protein gene (*OsRGLP1*) of rice origin was characterized by its heterologous expression in tobacco. The real-time PCR established almost a uniform expression of *OsRGLP1* in leaves, stem, and roots of T_1 *Nicotiana tabacum* cv. Samsun. Although no morphological difference was apparent between T_0 transgenic and wild-type plants, leaves of mature transgenic plants showed necrotic lesions associated with an elevated content of H_2O_2, which was evidenced by *in situ* 3,3'-diaminobenzidine staining. A significantly higher activity of heat resistant superoxide dismutase (SOD) was observed in the transgenic plants as compared to the wild-type. The SOD activity in the transgenic plants was insensitive to potassium cyanide and sensitive to H_2O_2.

Additional key words: hydrogen peroxide, *Nicotiana tabacum*, *Oryza sativa*, oxalate oxidase, superoxide dismutase.

Introduction

First germin protein was isolated from germinating wheat seeds (Thompson and Lane 1980) and since then a large number of germins and germin-like proteins has been identified from different plants of economic importance including rice, wheat, maize, and barley. Germins exist in oligomeric forms and are highly resistant to proteases and high temperature (Lane 1994, Dunwell *et al*. 2008). Proteins with sequence similarity (30 - 70 %) to germins have also been identified in diverse plant groups and are known as germin-like proteins (GLPs). Rice having a large family of germin-like protein genes presents a good model for studying the structural and functional diversity of these genes and their protein products. Members of the GPL family have been reported to express in various tissues (Godfrey *et al*. 2007, Komatsu *et al*. 2010, Wang *et al*. 2013) and under diverse environmental conditions (Yasmin *et al*. 2008, Banerjee and Maiti 2010, Galván *et al*. 2011, Yang *et al.* 2013).

The first germin was characterized in wheat by Lane (1994). It is a water soluble glycoprotein associated with the cell wall and it exhibits oxalate oxidase (OXO) activity (the conversion of oxalate to hydrogen peroxide). The majority of germins identified so far exhibit OXO activity, whereas GLPs generally have superoxid dismutase (SOD) activity (Dunwell *et al.* 2008). GLPs assemble into homohexameric complexes *in vivo* with a molecular mass of 100 - 120 kDa and are resistant to heat, detergents, and proteases (Vallelian-Bindschedler *et al*. 1998).

The expression of GLPs is quite diverse. Some are expressed at critical developmental stages, such as embryogenesis, early development, floral induction, or fruit ripening (Mathieu *et al*. 2006, El-Sharkawy *et al*. 2010). Many GLPs are induced by biotic and abiotic stresses, such as pathogens (Galván *et al*. 2011, Olvera-Guevara *et al*. 2012, Wang *et al*. 2013), herbivores (Lou and Baldwin 2006), drought, or salinity (Wang *et al*. 2013). The GLPs identified so far have been detected in different organs and tissues (Godfrey *et al*. 2007, Komatsu *et al*. 2010) including the cell wall. The presence of an *N*-glycosylation site in the sequence of almost all germins and GLPs reinforces their extracellular

Submitted 28 April 2014, *last revision* 24 February 2015, *accepted* 4 March 2015.
Abbreviations: CTAB - cetyldimethylammonium bromide; NBT - nitroblue tetrazolium; OXO - oxalate oxidase; SOD - superoxide dismutase.
Acknowledgments: We are grateful for a financial assistance to S.M.S. Naqvi (grant No. # 01-03-01-19 from the Agricultural Linkage Program of the Pakistan Agricultural Research Council).
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location (Kondo *et al*. 2008, Komatsu *et al*. 2010, Galván *et al*. 2011, Wang *et al*. 2013).

In spite of versatility in expression and wide distribution, GLPs do not seem to possess very diverse functions. Mostly they are associated with SOD activity (Carter and Thornburg 2000, Mathieu *et al*. 2006, Kondo *et al*. 2008, Galván *et al*. 2011, Olvera-Guevara *et al*. 2012). The exception is one GLP serving as allergen in *Citrus reticulata* (Ebo *et al*. 2007). The actual physiological role of GLPs has not been fully known yet; both OXO and SOD produce H_2O_2 which is signaling molecule implicated in various processes like induction of defense mechanism, cross-linking polymers in extracellular matrix, cell wall synthesis and reinforcement, *etc*. To our knowledge, there is only one report in which one out of about 30 rice GLP proteins has been characterized in detail (Banerjee and Maiti 2010). Therefore, the aim of the present study was to isolate and characterize one of the root specific *GLP* genes of rice (*OsRGLP1*) by its heterologous expression in tobacco.

Materials and methods

Cloning and construct designing: Cloning *OsRGLP1* full length complementary DNA (cDNA) was carried out according to Sambrook and Russell (2001). Total RNA obtained from roots of rice (*Oryza sativa* L.) cv. Nonabokra was used for cDNA synthesis. The cDNA of *OsRGLP1* was amplified with forward (5'-ATCTAGATCTCATCTCAAACACACCACC-3') and reverse (5'-CTCGAGGTGACCGTCACAAAGAAC ACTG-3') primers and ligated into pTZ57R/T (*Thermo Fisher Scientific*, Waltham, MA, USA) and electroporated into *Escherichia coli* strain DH5α (Janjua *et al.* 2014). Plasmid DNA (pTZ57R/T*Os*RGLP1) isolated from transformed *E. coli* was sequenced from *Macrogen* (Seoul, Korea) using universal primers M13 forward (5'-TGTAAAACGACGGCCAGT-3') and reverse (5'-CAGGAAACAGCTATGACC-3') present on either side of the multiple cloning site of pTZ57R/T.

To clone *OsRGLP1* cDNA in the sense direction under CaMV35S promoter in pCAMBIA1301, restriction sites of two enzymes, *i.e*., BglII and BstEII were introduced in 5' and 3'ends of *Os*RGLP1 with the help of RGLP1 forward (BglII 5'-ATCTAGATCTCATCT CAAACACACCACC-3') and RGLP1 reverse (BstEII 5'-CTCGAGGTGACCGTCACAAAGAACACTG-3') primers. The PCR product amplified on recombinant pTZ57R/T:*OsRGLP1* plasmid template was digested with BglII and BstEII and ligated in pCAMBIA1301 by T_4 DNA ligase at 16 ºC overnight and followed by electroporation into *Agrobacterium tumefaciens* strain EHA101. The orientation of the cDNA with respect to CaMV35S promoter was verified with the help of restriction digestion with BamH1 and Pst1.

Transformation of *Nicotiana tabacum:* Seeds of *N. tabacum* L. cv. Samsung were obtained from the National Agriculture Research Center (NARC), Islamabad, Pakistan, and grown in a greenhouse. Leaves from one to two months old plants were used for transformation by a leaf disk transformation method of Klee *et al*. (1987) with *A. tumefaciens* strain EHA101 harboring pCAMBIA1301*:OsRGLP1*. Control plants were produced by the same procedure, except that they were not subjected to *A. tumefaciens* and were not kept on a selective medium. Transgenic plants were selected on a medium with 0.1 mM hygromycin for 90 d before being transferred to soil. The plants were kept under controlled conditions (a temperature of 24 ± 1 °C, a relative humidity of 60 %, a 16-h photoperiod, and an irradiance of 30 - 40 μmol m^{-2} s^{-1}) for three months and then shifted to a greenhouse. Genomic DNA from control and transgenic tobacco was isolated by the cetyl dimethylammonium bromide (CTAB) method (Richards 1997). The PCR for re-confirmation of the transgene was carried out with a similar pair of primers (RGLP1F and RGLP1R) as used previously to confirm the transgenic plants after transformation.

Heterologous expression analysis using real-time PCR: The expression pattern of *OsRGLP1* gene in leaves, stems, and roots of 25 transgenic *N. tabacum* plants at two different developmental stages was monitored by real-time PCR. A *SensiMixPlus SYBR* kit (*Bioline*, London, UK) was used to perform real-time PCR according to the manufacturer's instructions. Thermal cycling conditions included pre-amplification denaturation at 95 °C for 10 min followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 62 °C for 30 s, and extension at 72 °C for 40 s. A *Line-Gene K fluorescence* quantitative PCR detection system (*Stratagen*, La Joll, CA, USA) was used to analyze data obtained from real-time PCR. For quantification of RNA expression, the $2^{-\Delta\Delta Ct}$ method was used (Schmittgen and Livak 2008) and the *actin* gene was used as internal control.

Hydrogen peroxide and protein estimation: H_2O_2 in wild type and transgenic leaves was detected using the 3,3'-diaminobenzidine (DAB) staining method according to Thordal-Christensen *et al*. (1997).

Protein content was determined by a protein-dye binding assay using bovine serum albumin (BSA) as standard (Bradford 1976).

OXO and SOD activities assay: The OXO activity was performed on mature and immature T_0 plants, germinating seeds, and early developmental stages of control and T_1 generation of transgenic tobacco plants. Tissue localization of OXO activity was determined according to the method established by Liang *et al.* (2001). Germinating seeds of wheat cv. Channab 2000

were used as positive control for OXO activity. Plant tissues were photographed with a digital camera (*DSF1*, *Nikon,* Tokyo, Japan).

Transgenic plants were evaluated for SOD activity as described by Dhindsa *et al*. (1981) with few modifications. Samples of control and transgenic tobacco leaves (1 g) were homogenized in a pre-cooled mortar and pestle in 3 cm^3 of a 0.2 M phosphate buffer, pH 7.0 at 4 °C and centrifuged at 20 000 *g* in a refrigerated centrifuge (*Sigma 2-15KC,* Osterode am Harz, Germany) for 30 min. The supernatant was used to estimate the activity of SOD by recording the decrease in absorbance of superoxide-nitroblue tetrazolium (NBT) complex. Two sets of cuvettes, each provided with about 3 cm^3 of a reaction mixture containing the enzyme extract (0, 50, 100, 200 mm^3), 13 mM methionine, 75 mM nitro-blue tetrazolium, 0.1 mM EDTA, 50 mM potassium phosphate buffer (pH 7.8), and 2 μM riboflavin, were prepared for control and transgenic plants. Two tubes without the enzyme extract were taken as controls. The reaction was started by placing the tubes under two 15 W fluorescent lamps for 15 min and then stopped by switching off the light and covering the tubes with black cloth. A non-irradiated complete reaction mixture that did not develop colour served as blank. Absorbance (A_{560}) was recorded with a *Shimadzu* spectrophotometer (Kyoto, Japan) at 25 °C, and one unit of enzyme activity was taken as the quantity of enzyme which reduced the absorbance of the samples to 50 % in comparison with the tubes lacking the enzymes.

Statistical analysis: The experiments were repeated three times with at least five samples in each trial. Two-way *ANOVA* followed by the least significant difference test was applied for comparison of enzyme assays in control and transgenic plants using the *MSTAT-C* software (Michigan State University 1988).

Results

The cDNA of rice root specific germin-like protein gene1 (*OsRGLP1*, accession No. AF141878) cloned in pTZ57R/T in *E. coli* strain DH5α was confirmed by restriction digestion and sequencing. The sequence of 482 nucleotides so obtained was identical to the 5' end of germin-like protein gene1 precursor of rice available in GenBank (acc. No. AF141878). This cDNA (*OsRGLP1*) was 958 bp long and capable of encoding a 224 amino acids long polypeptide. Signal peptide search using *SignalP 3.0* server predicted the presence of a typical extracellular targeting peptide of 22 amino acids at the N-terminus and a cleavage site between position 22 and 23

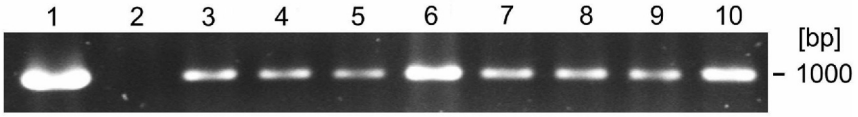

Fig. 1. Confirmation of *OsRGLP1* expression in *N. tabaccum* transgenic plants. PCR from pCAMBIA1301:*OsRGLP1* construct as positive control (l*ane* 1), genomic DNA of wild type tobacco as negative control (*lane* 2) and genomic DNA of different T_1 transgenic plants (*lanes* 3 to 10).

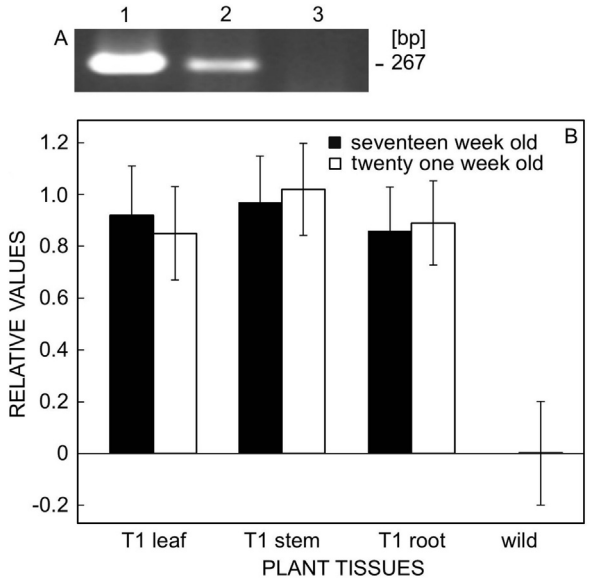

Fig. 2. The relative *OsRGLP1* expression in T_1 transgenic tobacco plants. *A* - Real time PCR of *OsRGLP1* amplification in pTZ57R/T as positive control (*lane* 1), a 1:100 dilution of cDNA from transgenic plant (*lane* 2), and a 1:10 dilution of cDNA from wild type tobacco as negative control (*lane* 3). *B* - Relative *OsRGLP1* expressions in leaves, stems, and roots of 17- and 21-week-old T_1 transgenic plants and in wild-type plants. Relative values (Ct) obtained by normalization to an *actin* gene expression. Means ± SE, $n = 3$.

A typical germin family signature (GqnppHtHPrAtEI) was found located at position 104 to 117 when predicted through *ScanProsite* server. The molecular mass of mature OsRGLP1 was predicted to be 24.2 kDa.

Directional cloning *OsRGLP1* cDNA was performed in BglII and BstEII enzyme sites in pCAMBIA1301 downstream CaMV35S promoter replacing 2 kb of the β-glucuronidase (*GUS*) gene. The orientation of cDNA with respect to the promoter in *pCAMBIA1301* was confirmed by restriction digestion of the recombinant plasmid with BamH1 and Pst1, which yielded a 1 kb fragment

confirming the orientation of the insert in sense direction. The confirmed recombinant plasmid was electroporated into *A. tumefaciens* strain EHA101 and subsequently used for tobacco plant transformation. The *OsRGLP1* amplification was con-firmed by PCR of genomic DNA of the transgenic plants with the same set of primers as used earlier. This yielded a 1 kb fragment of *OsRGLP1* in genomic DNA samples obtained from many transgenic plants, whereas samples from control plants did not yield any band (Fig. 1).

Almost a uniform expression of *OsRGLP1* in leaves, stems, and roots of the transgenic plants was observed using a real-time PCR analysis. The uniform expression could be attributed to the transcriptional control of *OsRGLP1* cDNA under CaMV35S promoter in the transgenic plants (Fig. 2).

The presence of H_2O_2 in the transgenic plants was detected by the DAB staining method. Leaf sections from the transgenic plants demonstrated higher DAB staining as compared to the non-transgenic controls. In the

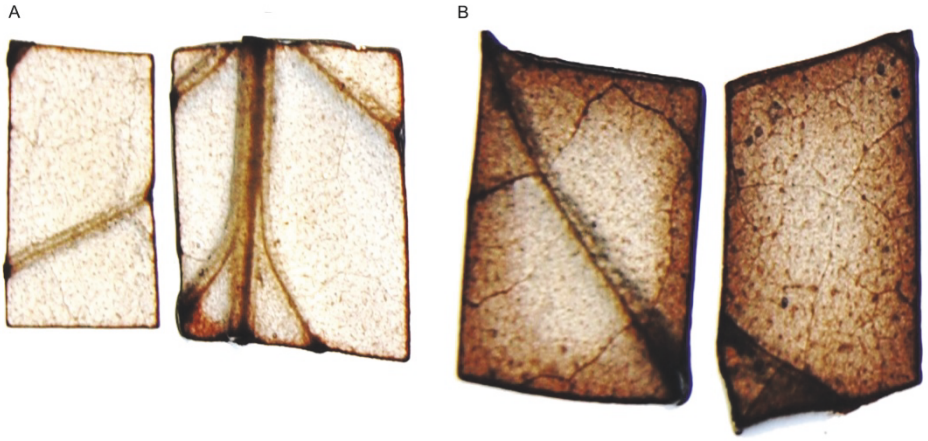

Fig. 3. Detection of hydrogen peroxide in tobacco leaves through DAB staining (*A* - controls, *B* - T_1 transgenic plants).

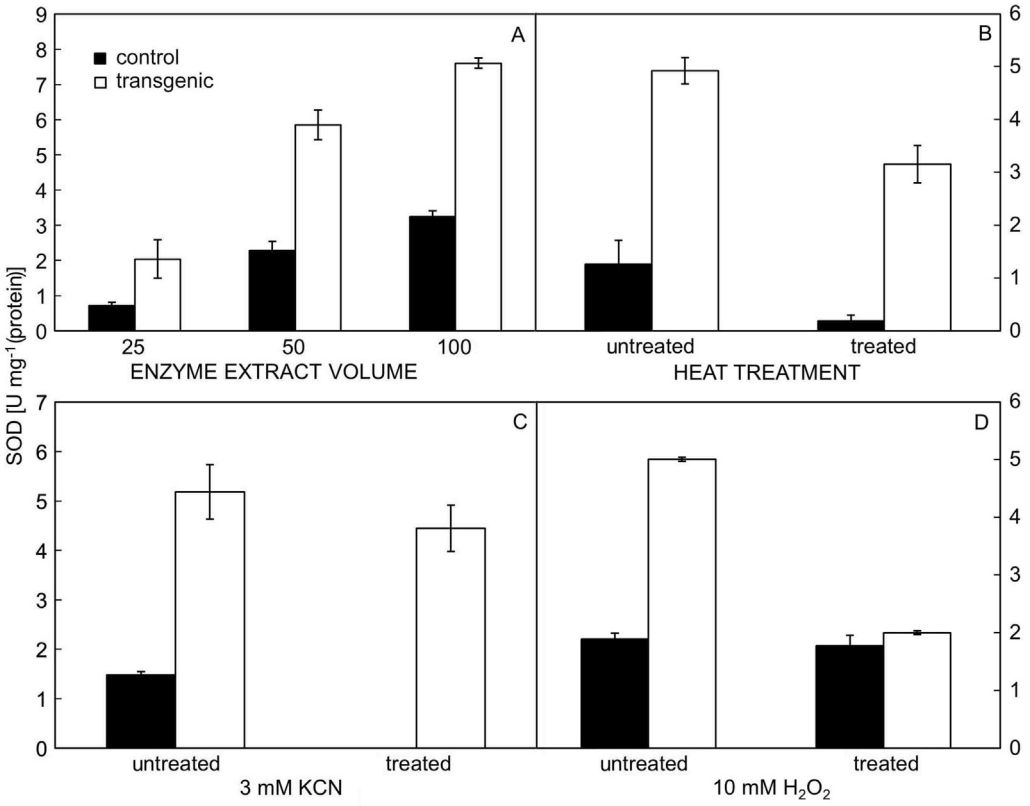

Fig. 4. SOD activity in leaf extracts from control and T_1 transgenic tobacco plants. The effects of extract volume [mm^3] (*A*), the effects of heat (*B*), KCN (*C*), and H_2O_2 (*D*) on SOD activity. SOD activity was determined using 50 mm^3 of the extract except in *A* where different volumes were used as indicated. Means ± SE, $n = 3$. Differences between transgenic and control plants are significant at $P < 0.05$.

absence of an OXO activity and in the presence of an elevated SOD activity in the transgenic plants, this additional DAB staining in the transgenic plants might be attributed to the dismutation of superoxide anions to H_2O_2. Hence, it seems evident that the *OsRGLP1* transgenic plants constitutively expressed SOD as evidenced by DAB staining H_2O_2 (Fig. 3).

No detectable OXO activity was observed in leaf cuttings of the control and mature T_0 and T_1 transgenic plants, whereas wheat seeds used as positive control showed a strong blue colour on the germinating seed surface. Six months old T_0 transgenic plants were evaluated for SOD activity using leaf extracts. Leaf extracts of the T_0 transgenic plants showed a significantly enhanced SOD activity as compared to the control. Similarly, a significantly higher SOD activity was obtained from leaf extracts of three months old T_1 transgenic plants (Fig. 4*A*).

In order to know whether the SOD activity in the transgenic plants was heat tolerant or heat sensitive, the leaf extracts from the transgenic and control plants were heated at 90 °C for 15 min prior to the assay. After the heat treatment, the SOD activity of the transgenic samples remained significantly higher than that of the control samples (Fig. 4*B*). The consitutive SOD activity in the control plants registered a 90 % decrease indicating that the constitutive SOD activity in *N. tabacum* is mainly not connected with GLPs. In the case of the transgenic plants after subtracting the constitutive activity, the SOD activity was 4-fold higher and on heating decreased only by about 3 % (data not shown). This clearly points out that an additional SOD activity gained by the transgenic plants was due to the heat resistant SOD and thus might be due to *OsRGLP1*.

Both KCN and H_2O_2, which are known inhibitors of different SODs, were used to study the nature of the transgenic SOD. The transgenic and control samples were treated with 3 mM KCN before the SOD assay. There was no significant difference in the SOD activity in the treated and untreated transgenic samples, whereas the SOD activity was abolished in the control samples treated with KCN indicating the presence of KCN sensitive Cu/ZnSOD in the control samples. An additional SOD activity in the untreated transgenic samples was 3-fold higher than control (Fig. 4*C*). This SOD activity observed in the transgenic samples appeared insensitive to KCN suggesting that it was not Cu/ZnSOD.

To find out the effect of H_2O_2 on SOD activity, the transgenic and control samples were treated with 10 mM H_2O_2 before the SOD assay. It was observed that the SOD activity of the treated transgenic samples significantly decreased compared to the untreated samples, whereas in the control samples, this activity remained unaffected (Fig. 4*D*). These findings suggest that an increased SOD activity in the transgenic plants was H_2O_2 sensitive, a behavior similar to bacterial FeSOD.

Discussion

Sequence information from the rice genome indicates that the *GLPs* constitute a multigene family with more than 30 genes. Although the expression of different members of the family has been documented under a number of biotic and abiotic stresses, until recently only few studies have been directed to explore their biological activity (Banerjee and Maiti 2010, Gambino *et al.* 2013). About 12 *GLP* genes associated with the rice blast disease resistance were found as cluster on chromosome 8 (Manosalva *et al*. 2009) showing the involvement of *GLPs* in defense. The present study was conducted to characterize one of these *GLP* genes found on chromosome 8, and its product expressed in rice roots. The *OsRGLP1* cDNA was cloned, transformed into tobacco, and functionally characterized. The T_0 and T_1 transgenic plants harboring the full length cDNA of *OsRGLP1* were similar to the wild-type plants in morphology; however unlike control, the six months old transgenic plants showed hypersensitive response-like necrotic lesions on older leaves when kept at day/night temperatures of 30/24 °C and under direct sunlight in the absence of a challenge with any microbe. As GLPs are connected with OXO and/or superoxide dismutase activities resulting in production of H_2O_2 (Gambino *et al.* 2013), it was speculated that the product of *OsRGLP1* may possess either one or both activities and generate H_2O_2 resulting in hypersensitive reaction (HR)-like lesions under a high temperature and sunlight. When leaf disks from the transgenic and control plants were subjected to DAB staining, the transgenic leaf disks exhibited a higher content of H_2O_2. The HR-like lesions have also been observed by Hu *et al*. (2003) on mature leaves of transgenic sunflower plants constitutively expressing wheat OXO gene. Further, these lesions were not associated with senescence of tobacco which started much later as observed in the wild type plants in the present study. Moreover, there are many reports where hypersensitive response or necrotic lesions have been observed in transgenic plants with an elevated content of H_2O_2 either due to a reduced catalase activity (Chamnongpol *et al*. 1996) or an enhanced activity of H_2O_2 producing enzymes (Hu *et al*. 2003, Olvera-Guevara *et al*. 2012).

In the present study, no OXO activity was detected in the transgenic or control plants in the assay in which germinating wheat seeds clearly showed an OXO activity. The absence of any OXO activity in leaves, stems, and roots of the transgenic tobacco over-expressing *OsRGLP1* is in accordance with a previous finding (Kim *et al*. 2004) concerning another *GLP*. Similarly, a GLP isolated from cells of *Barbula unguiculata* is connected with MnSOD activity but not with OXO activity (Yamahara *et al*. 1999). A significantly higher SOD activity was noted in the transgenic

tobacco (both T_0 and T_1 plants) expressing *OsRGLP1* in this study. However, plants contain different SODs (Wang *et al*. 2013), which was also evident in the control tobacco plants. As *GLPs* are heat resistant proteins (Vallelian-Bindschedler *et al*. 1998, Mathieu *et al*. 2006), this property was exploited to differentiate between the putative transgene directed SOD activity from the constitutive SOD activity. The results demonstrate a significantly higher retention of SOD activity in the heat treated transgenic samples as compared to control. This experiment suggests that the additional SOD activity in the transgenic samples might be due to the *OsRGLP1* expression. This result also reinforces the previous findings about the heat stability of GLPs.

It has now become evident that almost all the GLPs studied to date possess SOD activity (Banerjee and Maiti 2010, Galván *et al*. 2011, Olvera-Guevara *et al*. 2012), and OsRGLP1 also seems to possess this activity. This is reported for the first time that the rice root-specific GLPs exhibit SOD activity. In addition, all the reported SOD activities associated with GLPs are MnSODs (Yamahara *et al*. 1999), whereas in the present study, it seems to be FeSOD.

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