ACTA ABP BIOCHIMICA POLONICA

Vol. 70, No 1/2023
175–181
https://doi.org/10.18388/abp.2020_6441

Regular paper

Molecular cloning of glutathione reductase from *Oryza sativa*, demonstrating its peroxisomal localization and upregulation by abiotic stresses

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Abiotic stress is a major constraint on crop productivity and in the agricultural field, multiple abiotic stresses act synchronously leading to substantial damage to plants. A common after-effect of abiotic stress-induced damage in plants is an increased concentration of reactive oxygen species (ROS) leading to oxidative damage. Glutathione reductase (GR) plays a significant role in curtailing ROS. Apart from the GR enzyme, the peroxisome as an organelle also plays a significant role in ROS homeostasis. Here, we report a peroxisome localized GR, whose expression was found to be upregulated by various abiotic stresses. The *in silico* analysis also revealed that the peroxisomal localization of GR could be a common phenomenon in angiosperms, suggesting that it could be a suitable candidate against abiotic stress combinations.

Keywords: glutathione reductase, abiotic stress, stress combination, peroxisome, PTS

Received: 26 July, 2022; revised: 01 December, 2022; accepted: 05 December, 2022; available on-line: 08 January, 2023

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Acknowledgments of Financial Support: The financial support from the Science and Engineering and Research Board (SERB), Govt of India, and KIIT University is acknowledged.
Abbreviations: At, *Arabidopsis thaliana*; BLAST, basic local alignment search tool; CaMV, cauliflower mosaic virus; CT, cycle threshold; EYFP, enhanced yellow fluorescent protein; GR, Glutathione reductase; HT, high temperature; LT, low temperature; PCR, polymerase chain reaction; NCBI, National Center for Biotechnology Information; Os, *Oryza sativa*; PEX, Peroxin; PTS, peroxisomal targeting signal; ROS, reactive oxygen species; TAIR, The Arabidopsis Information Resource

INTRODUCTION

Abiotic stress is one of the significant factors which limits the yield of crops. Due to the untimely onset of abiotic stress conditions, crops worth millions of dollars are lost annually worldwide (Wang *et al.,* 2003; Wania *et al.,* 2016). Abiotic stress leads to a series of detrimental activities in the plant, which cause either partial or complete loss of yield (Yousuf *et al.,* 2012; Harshavardhan *et al.,* 2017). One such after-effect is an increase in the cellular concentration of reactive oxygen species (ROS), which has been observed as a common phenomenon in the case of all types of abiotic stresses. Uncontrolled increase and accumulation of ROS can lead to oxidative damage to the cell, such as oxidation of nucleic acids, protein denaturation, breakdown of cell and organelle membrane, lipid peroxidation, and carbohydrate oxidation (Scandalios, 1993; Noctor & Foyer, 1998). This imbalance in the redox state of the cell is dangerous and could be lethal if left unchecked. ROS also plays a significant role in regulating the response of plants, against environmental stimuli by a redox-dependent reprogramming of signalling pathways (Harshavardhan *et al.,* 2017), thereby making ROS not only an oxidative deterrent but also a signalling molecule (Mittler *et al.,* 2004; Miller *et al.,* 2010). Hence, a delicate balance between ROS production and scavenging needs to be maintained, making ROS homeostasis very crucial. To defend themselves from this abiotic stress-induced oxidative damage, plants have developed various mechanisms. One such mechanism is the glutathione reductase (GR) mediated ascorbate-glutathione pathway, also known as the Foyer-Halliwell-Asada pathway (Asada, 2006; Foyer & Noctor, 2011). In plant cells, GR activity has primarily been found in the chloroplast (70–80%) with a minor presence in mitochondria and cytosol (Edwards *et al.,* 1990; Creissen *et al.,* 1994). However, with recent development in molecular and proteomic techniques, the peroxisomal localization of *Arabidopsis thaliana* GR has also been demonstrated (Kataya & Reumann, 2010). Also, it is pertinent to mention that the peroxisomes in association with chloroplast and mitochondria are primarily responsible for cellular ROS homeostasis (Foyer & Noctor, 2003; Habib *et al.,* 2016; Dietz *et al.,* 2016; Huang *et al.,* 2016). The significant role of peroxisomes in ROS scavenging and abiotic stress tolerance makes a peroxisome-localized GR of special significance.

Peroxisomes are small, single membrane-bound organelles whose proteome is encoded by nuclear genes, synthesized on cytosolic ribosomes, and imported in a signal-dependent manner (Hu *et al.,* 2012; Emmanouilidis *et al.,* 2016). Depending upon the protein location, peroxisomal proteins could be divided into two broad categories- peroxisomal membrane and matrix proteins, both of which are imported in entirely different manners. GR happens to be a peroxisomal matrix protein (Kataya & Reumann, 2010). The peroxisomal matrix proteins are largely imported either by peroxisomal targeting signal (PTS) type one or PTS type two (Gould *et al.*, 1987; Swinkels *et al.*, 1991). The bulk of the matrix proteins is imported by PTS type 1 while a comparatively smaller number of matrix proteins are imported by PTS type 2 (Brocard & Hartig, 2006; Lazarow, 2006). After being synthesized on cytosolic ribosomes, PTS1 and PTS2 containing proteins are recognized by their respective cytosolic receptors, PEX (peroxin) 5 and PEX7 respectively, which ultimately bring them to peroxisome with the help of other PEX proteins (Kunze, 2019; Kim & Hettema, 2015; Kunze *et al.,* 2011; Kiel *et al.,* 2009; Niederhoff *et al.,* 2005; Reumann, 2004; Bottger *et al.,* 2000; Lametschwandtner *et al.,* 1998; Albertini *et al.,* 1997). The PTS1 is located at the C-terminus of protein and

is primarily represented by the last three amino acids, however, the upstream residues also play a significant role in PEX5 binding (Neuberger *et al.,* 2003; Brocard & Hartig, 2006; Lingner *et al.,* 2011; Lametschwandtner *et al.,* 1998; Reumann, 2004; Fodor *et al.,* 2012; 2015), while the PTS2 is located at N-terminus, represented by a nonapeptide (Petriv *et al.,* 2004; Lazarow, 2006). It has also been observed that depending upon the composition of amino acids present at the C-terminus, the PTS1 shows a varying degree of efficiency in peroxisome targeting (Gatto *et al.,* 2000; Stanley *et al.,* 2006) and this has led to the classification of PTS1 into a canonical and noncanonical type (Skoulding *et al.,* 2015). The canonical ones lead to efficient and strong targeting while the noncanonical ones are comparatively less efficient and lead to weak targeting of the protein to the peroxisome. The strong and weak efficiencies have been explained based on the time required for the fluorescent detection of the peroxisome targeting under *in vitro* conditions (Skoulding *et al.,* 2015; Chowdhary *et al.,* 2012).

In this work, we reported the peroxisome localization of GR and its involvement in abiotic stress tolerance in the monocot model plant *Oryza sativa* (rice) which is a crop of global significance and is responsible for feeding more than half of the world's population, especially in Asian, African continents, and other third world nations.

MATERIALS AND METHODS

Data retrieval for *in silico* work

The protein sequences of *A. thaliana* and *O. sativa* GR were retrieved from The Arabidopsis Information Resource (TAIR, Tanya *et al.,* 2015, https://www.arabidopsis.org/) and the rice genome annotation database (Kawahara *et al.,* 2013, http://rice.uga.edu/) respectively. For multiple sequence alignment, CLUSTAL W 2.1 program (Larkin *et al.,* 2007) was used at pir.georgetown.edu (Cathy *et al.,* 2003). The OsGR was used as a query in protein BLAST at NCBI and 80 GR orthologs were obtained.

Plant material and growth condition

For all experiments, *O. sativa* IR 64 (Indica rice) variety was used. This is an abiotic stress-sensitive variety. The seeds were obtained from National Rice Research Institute, Cuttack, India. The seeds were sterilized with 70% ethanol for 2 min followed by rinsing with distilled water three times each for 2 min. The sterilized seeds were kept in germination paper pre-wet with distilled water, which was incubated in dark for 24 h followed by shifting to the plant growth room maintained at 28±2ºC, 60% humidity, and a light/dark cycle of 16/8 h. For all experimentation, 11 days old seedlings were used. The abiotic stresses were induced by treatment with 200 mM sodium chloride (saline stress), 4±2°C (cold stress), and 45±2°C (heat stress) for 2 h. The untreated seedlings served as a control and were used for the calculation of the relative transcript level of *OsGR*.

RNA extraction and cloning of *OsGR*

For RNA extraction, 100 mg of seedling was used and crushed in liquid nitrogen followed by extraction using RNeasy plant mini kit (Qiagen) as per the manufacturer's protocol, with the modification of inclusion of in-column DNase digestion step (RNase free DNase set, Qiagen). The extracted RNA was checked for its quantity and quality using a microvolume spectrophotometer (ThermoFisher Scientific) and agarose gel electrophoresis.

The extracted RNA was converted to cDNA using Revert Aid First Strand cDNA Synthesis Kit (ThermoFisher Scientific) as per the manufacturer's manual. The obtained cDNA was used for polymerase chain reaction (PCR) amplification using the primers: Forward AATTGCGGCCGCGATGGCTAGGAAGATGCTCAAG, Reverse TATGTCTAGAGCTACAAGTTTGTCTTTGGCTTGGATGATGG, using HiFidelity polymerase (Qiagen), with PCR cycle of denaturation 94ºC for 20 s, annealing 60ºC for 30 s and extension 72ºC for 90 s. The obtained PCR product was checked for amplification using agarose gel electrophoresis followed by digestion using NotI and XbaI restriction enzymes. The digested product was resolved in an agarose gel followed by gel purification using the GenJET gel extraction kit (ThermoFisher Scientific). The digested product was cloned into the pCAT plant expression vector, as an enhanced yellow fluorescent protein (EYFP) fusion product under the control of a double 35 S cauliflower mosaic virus (CaMV) promoter (Fulda *et al.,* 2002). The insert was verified using automated DNA sequencing.

Subcellular localization of OsGR

For subcellular localization studies, the biolistic bombardment method was used, in which onion epidermal cells were transformed with plasmid constructs coated on gold particles, which were further examined under a fluorescent microscope after an appropriate incubation time. Under standard conditions, the transformed onion epidermal cells are examined after 18–24 h post-transformation. However, it has also been observed that the sensitivity of detection of the reporter protein in peroxisome increases after an extended incubation time at reduced temperature (Lingner *et al.,* 2011, Chowdhary *et al.,* 2012).

The gene of interest (*OsGR*) was cloned as an EYFP fusion product and as a peroxisomal marker, Ds-Red-SKL was used (Matre *et al.,* 2009). In the case of single transformation experiments pCAT plasmid containing EYFP-OsGR was coated with gold particles, while in the case of double transformation experiments pCAT plasmid containing EYFP-OsGR and peroxisomal marker (Ds-Red-SKL) together were coated with gold particles and bombarded to onion epidermal cells (Ma *et al.,* 2006). The onion slices were placed on wet blotting paper in Petri dishes and stored at room temperature in the dark followed by analysis using fluorescent microscopy or after additional incubation at 10°C for 1 to 6 d. Image capture and analysis were done as explained in Chowdhary *et al.,* (2012).

Expression analysis by real-time PCR

The expression analysis of peroxisomal GR was carried out by real-time PCR technique using SYBR green chemistry. For this, the total RNA was extracted from stress-treated and control (untreated) plants as explained above, its concentration was determined, equalized, and converted to cDNA using high-capacity cDNA reverse transcription kit (ThermoFisher Scientific) as per the manufacturer's manual. Total RNA, 1 µg, was converted to cDNA, which was diluted and further used for quantitative real-time PCR using SYBR green chemistry. Real-time PCR was performed using QuantiNova 2X PCR master mix containing SYBR green dye with ROX as passive reference dye (Qiagen) in QuantStudio 5 (ThermoFisher Scientific) real-time PCR

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Os  MARKMLKDEEVEVAVTDGG---SYDYDLFVIGAGSGGVRGSRTSASFGAKVAICELPFHP
AT  MARKMLVDGEIDKVAADEANATHYDFDLFVIGAGSGGVRAARFSANHGAKVGICELPFHP

Os  ISSDWQGGHGGTCVIRGCVPKKILVYGSSFRGEFEDAKNFGWEINGDINFNWKRLLENKT
AT  ISSEEIGGVGGTCVIRGCVPKKILVYGATYGGELEDAKNYGWEINEKVDFTWKKLLQKKT

Os  QEIVRLNGVYQRILGNSGVTMIEGAGSLVDAHTVEVTKPDGSKQRYTAKHILIATGSRAQ
AT  DEILRLNNIYKRLLANAAVKLYEGEGRVVGPNEVEVRQIDGTKISYTAKHILIATGSRAQ

Os  RVNIPGKELAITSDEALSLEELPKRAVILGGGYIAVEFASIWKGMGAHVDLFYRKELPLR
AT  KPNIPGHELAITSDEALSLEEFPKRAIVLGGGYIAVEFASIWRGMGATVDLFFRKELPLR

Os  GFDDEMRTVVASNLEGRGIRLHPGTNLSELSKTADGIKVVTDKGEEIIADVVLFATGRTP
AT  GFDDEMRALVARNLEGRGVNLHPQTSLTQLTKTDQGIKVISSHGEEFVADVVLFATGRSP

Os  NSQRLNLEAAGVEVDNIGAIKVDDYSRTSVPNIWAVGDVTNRINLTPVALMEATCFSKTV
AT  NTKRLNLEAVGVELDQAGAVKVDEYSRTNIPSIWAVGDATNRINLTPVALMEATCFANTA

Os  FGGQPTKPDYRDVPCAVFSIPPLSVVGLSEQQALEEAKSDVLVYTSSFNPMKNSISKRQE
AT  FGGKPTKAEYSNVACAVFCIPPLAVVGLSEEEAVEQATGDILVFTSGFNPMKNTISGRQE

Os  KTVMKLVVDSETDKVLGASMCGPDAPEIIQGMAVALKCGATKATFDSTVGIHPSAAEEFV
AT  KTLMKLIVDEKSDKVIGASMCGPDAAEIMQGIAIALKCGATKAQFDSTVGIHPSSAEEFV

Os  TMRTLTRRVSPSSKPKTNL
AT  TMRSVTRRIAHKPKPKTNL
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OsGR AHKPKPKTNL PEX5

Figure 1. Multiple sequence alignment of GRs from *Arabidopsis thaliana* and *Oryza sativa*.
GR sequences were obtained from the database and aligned using CLUSTAL W 2.1. "*"represents the same amino acids, ":"represents the similar amino acids present in both the sequences. The red rectangle highlights the PTS1 tripeptide with seven upstream residues. The figure at the bottom shows the diagrammatic representation of the interaction of PEX5 with the C-terminus end of GR. The solid black and blue rectangle represents the OsGR and PEX5, respectively.

machine. The conditions for real-time PCR were as follows: initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 60°C for 60 s, and 72°C for 60 s. To obtain the melting curve a program of 95°C for 15 s and 60°C for 1 min was followed. All qPCR analyses were performed in three biological replicates. *O. sativa* actin was used as an endogenous control for the normalization of the transcript. The relative transcript level or "fold change" (degree of change in expression between the treated sample and untreated sample) was calculated by the ΔΔCT method using the formula, relative transcript level/fold change = $2^{-\Delta\Delta CT}$, where ΔΔCT = ΔCT test sample – ΔCT calibrator sample (ΔCT test sample = CT test – CT reference, ΔCT calibrator = CT calibrator – CT reference; test sample = stress treated sample, calibrator = untreated sample, reference = endogenous control) (Livak & Schmittgen, 2001). The data provided represents an average of three biological replicates of independent samples and the values depicted represent mean ± standard deviation. The primer used for expression analysis were, *OsGR* Forward primer – CGACCTTTGACAGCACTGTTGG, reverse primer – TGGTCAAGGTCCGCATTGTCAC, OsActin forward primers – CAGCCACACTGTCCCCATCTA, Reverse primer – AGCAAGGTCGAGACGAAGGA.

RESULTS

Bioinformatics analysis of OsGR

The protein sequence of *A. thaliana* and *O. sativa* GR were aligned using CLUSTAL W 2.1 and the alignment revealed the significant conservation among both sequences (Fig. 1). The peroxisome targeting of *A. thaliana* GR is dependent on the PTS type 1, which is localized at the C-terminus of the protein. In the case of AtGR the PTS1 is represented by TNL> (Kataya & Reumann, 2010, the symbol ">" represents the end of the polypeptide). The presence of the same tripeptide in *O. sativa* GR provided a hint for peroxisomal localization of OsGR *via* PTS1 (Fig. 1). Hence, OsGR was fused at the C-terminus of the EYFP, so that the targeting signal is freely available for the cytosolic receptor, PEX5 binding followed by peroxisome targeting (Fig. 1).

Molecular cloning and subcellular localization of OsGR

The cDNA of the *O. sativa* was used as a template for PCR-based cloning of GR. The primer sequences and PCR cycle are explained in the materials and method section. The PCR amplified product was found to be of 1500 bp and was cloned in the pCAT vector under the control of a double 35 S CaMV promoter. The gene was cloned as a C-terminal fusion of enhanced yellow fluorescent protein (EYFP, Fig. 1). The sequence of the cloned gene was verified using automated DNA sequencing. No mutations were detected.

Onion epidermal cells transformed with EYFP, which served as a negative control showed uniform cytosolic fluorescence (Fig. 2A). The OsGR, which was expressed as a fusion protein with EYFP, showed green cytosolic fluorescence after 24 h of incubation (Fig. 2B1), however the same was observed in small punctuate structures after an extended time of incubation (Fig. 2B2). The identity of fluorescing punctuate structures was confirmed by double transformation experiments co-expressing peroxisomal marker DsRed-SKL. The double transformation experiments demonstrated the overlapping of both green and red fluorescence from

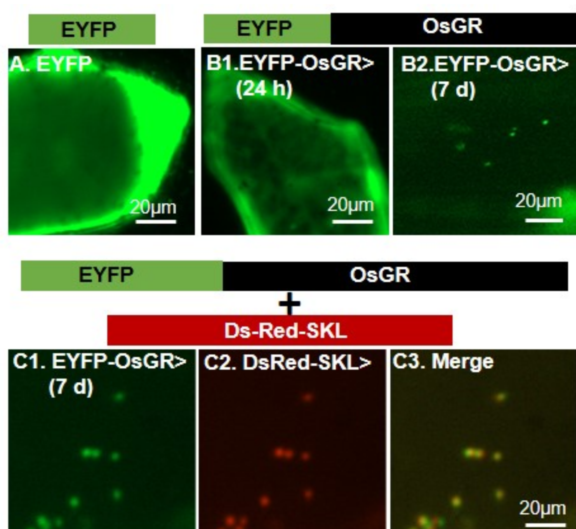

Figure 2. Experimental validation of peroxisome localization of *Oryza sativa* GR by *in vitro* subcellular targeting.
The *OsGR* was cloned in the pCAT plant transient vector under the control of a double 35 S CaMV promoter. The plasmids were coated with gold particles and bombarded biolistically to onion epidermal cells. The onion epidermal cells were incubated in dark for 24 h at room temperature or low temperature for an extended time period (24 h RT plus 6 d cold ca. 10°C) followed by analysis using fluorescent microscopy. EYFP alone was included as negative control (**A**). 24 h p.t., EYFP fluorescence was detected in cytosol only (**B1**), while after extended incubation the EYFP fluorescence was detected in punctuate structures (**B2**). The identity of the dot-like punctuate structure was confirmed by double transformation experiments, where peroxisomes were labelled with Ds-Red-SKL. The green (**C1**) and red fluorescence (**C2**) from EYFP and Ds-Red-SKL> respectively merged to show yellow fluorescence (**C3**) confirming the punctuate structure to be peroxisomes. The green rectangle bar fused to black depicts the diagrammatic representation of OsGR fused at the C-terminus of EYFP, while the red rectangular bar represents the Ds-RED-SKL>. The upper and bottom halves of the image show the single and double transformation experiments respectively.

EYFP and peroxisomal marker respectively, (Fig. 2C), confirming the identity of the punctuate fluorescing organelle to be peroxisome. Since fluorescence could not be detected in peroxisome 24 h post-transformation and it took an extended time of incubation for the fluorescence to be detected in peroxisome it was presumed that the GR is targeted to peroxisome with lesser efficiency and hence the PTS1 present in GR was termed as weak or non-canonical PTS1.

Expression analysis of *OsGR*

For *OsGR* gene expression analysis, the abiotic stress-treated seedlings were used for RNA extraction, followed by cDNA synthesis and real-time PCR. The untreated seedlings were used as a control. The expression data were represented as relative transcript level or "fold change" which essentially means the change in the degree of expression between the treated sample and untreated samples. The details are explained in the materials and method section. Expression analysis was performed with respect to heat, cold, and salinity (sodium chloride) stresses. All the treatments were provided for 2 h. In the case of heat stress, the change in the transcript accumulation was observed to a tune of 3.1-fold (Fig. 3), meaning that GR gene transcripts accumulated 3.1 times more in the heat stress as compared to untreated samples. This was followed by cold and saline stress with transcript level accumulation of about 3.7 and 10.4-fold respectively (Fig. 3). This demonstrated that the expression of peroxisomal

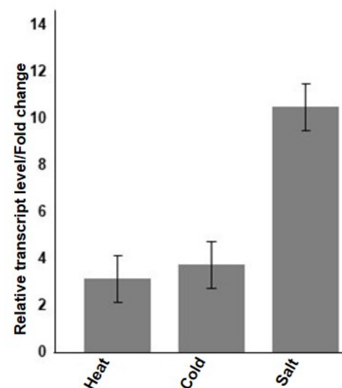

Figure 3. Expression analysis of *OsGR*.
The expression analysis was performed using the RNA isolated from *Oryza sativa* seedlings after the treatments. RNA extraction was done by RNeasy plant mini kit, (Qiagen) followed by cDNA synthesis (High-Capacity cDNA Reverse Transcription Kit, ThermoFisher Scientific). The cDNA obtained was used for expression analysis by real-time PCR using SYBR green chemistry. OsActin was used as endogenous control. The assays were repeated with a minimum of three replicates and ΔΔCT values were calculated, relative transcript level (fold change) was determined, and the graph was plotted. The x and y-axis show the various abiotic stress treatments and fold change, respectively. The data represent three biological replicates and error bars have been shown.

localized GR in *O. sativa* upregulated in the presence of heat, cold, and salinity stress conditions.

DISCUSSION

Abiotic stress is the major bottleneck in the sustainability of the global agricultural system. The harmful effect of abiotic stress is further compounded when more than one abiotic stress factor negatively affects the plant in synchrony, which is more often the case in field conditions. This phenomenon has been termed stress combinations (Zandalinas *et al.* 2020; 2021) or stress matrix (Mittler, 2006). This necessitates the development of crop varieties that would be tolerant to multiple abiotic stresses. Hence, candidate genes are required which would be effective against most if not all the abiotic stresses. Since the production of ROS is a common factor among all abiotic stresses, we intend to investigate the problem of abiotic stress combinations from the perspective of ROS homeostasis.

Since, peroxisomes play a significant role in cellular ROS homeostasis, a peroxisome-localized candidate participating in ROS homeostasis would be of greater significance. Using *in vitro* subcellular localization techniques, we reported a GR isoform from *Oryza sativa* to be localized in peroxisomes. Previously, Kataya & Reumann, (2010) have reported the localization of *A. thaliana* GR in peroxisomes. The peroxisome targeting of GR is mediated *via* a non-canonical type of PTS1. The PTS1 tripeptide of GR is represented by TNL> in both *A. thaliana* (Kataya & Reunann, 2010) and *O. sativa*. In the case of non-canonical PTS1, the seven upstream residues also play a significant role in binding with the cytosolic receptor PEX5 (Fodor *et al.,* 2012; Brocard & Hartig, 2006; Lingner *et al.,* 2011), which is represented by SPSSKPKTNL> and AHKPKPKTNL> (underlined residues PTS1 tripeptide, others, seven upstream residues) in the case of *O. sativa* and *A. thaliana* respectively. Being a non-canonical type of PTS1, the targeting is not very efficient and requires an extended incubation time for the fluorescence to be detected in the peroxisome (Lingner *et al.,* 2011; Chowdhary *et al.,* 2012). To further understand the peroxisome targeting signal of GRs in angiosperms we

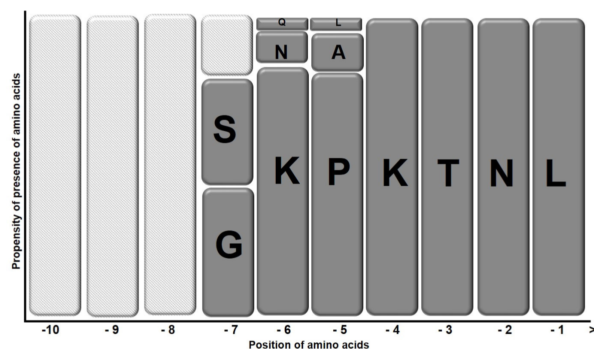

Figure 4. Graphical representation of PTS1 domain present in GR of various angiosperm plants.
The 80 numbers of GR sequences from various dicot and monocot plant species were obtained from NCBI. The sequences were aligned using CLUSTAL W 2.1 (Supplementary Fig. 1 at https://ojs.ptbioch.edu.pl/index.php/abp/). From the multiple sequence alignment, the consensus amino acids were derived. The y-axis shows the propensity of the presence of specific amino acid at a specific position while the x-axis represents the position of amino acids in the C-terminus of the respective protein. ">" denotes the end of the polypeptide chain. Each square represents one amino acid. The bigger the size of the squares, the higher the propensity of amino acids to remain present at that specific position. The empty squares represent the high variability.

obtained GR protein sequences from 80 plant species, and the last ten amino acids were aligned by multiple sequence alignment (Supplementary Fig. 1 at https://ojs.ptbioch.edu.pl/index.php/abp/). The alignment revealed that all the sequences were terminating with TNL> suggesting that the presence of a peroxisomal GR could be a common feature among angiosperms. At the -4 position, all the 80 GR orthologs have lysine while at the -5 position 73 sequences have proline except six sequences, one each from *Elaeis guineensis*, *Gossypium hirsutum*, *Gossypium Raimondi*, *Amborella trichopoda*, *Cinnamomum micranthum,* and *Phoenix dactylifera* have alanine and one sequence namely *Brachypodium distachyon* had leucine. At the -6 position, 75 sequences had lysine while asparagine was present at four places (one sequence each from *Citrus clementina*, *Olea europaea* and two sequences from *Citrus sinensis*) and glutamine was present at one place (*Phoenix dactylifera*). The most favourable amino acid at the -7 position is glycine which is present in 35 sequences followed by serine present in 29 sequences. At -8, -9 and -10 positions variability increases. The analysis curtailed here reveals that the consensus PTS1 sequence for peroxisomal GRs present in the angiosperms would be [G/S/T/N/A/V] [L/N/Q] [P/A/L] [K] [T] [N] [L]> at -7, -6, -5, -4, -3, -2 and -1> positions, respectively. Figure 4 demonstrates the diagrammatic representation of the propensity of specific amino acids to remain present at specific positions.

Further, the expression pattern of peroxisomal GR was investigated under various abiotic stress conditions. It was found to be upregulated upon heat, cold, and salinity stress in increasing order. GR expression has been reported to get affected due to stress conditions in various plant species, however, all previous reports are of either chloroplastic or cytosolic variants. Salinity and drought stress-dependent upregulation of GR has been demonstrated in *Cicer arietinum* and *O. sativa* respectively (Yousuf *et al.,* 2012). A positive correlation between tolerance to low temperature (LT)-induced photoinhibition and high GR activities has been observed in *O. sativa* (Guo *et al.,* 2006; Huang & Guo, 2005), *C. sativus* (Hu *et al.,* 2008), (Xu *et al.,* 2008), *Glycine max* (Sun *et al.,* 2011), *Cucumis melo* (Fogelman *et al.,* 2011) and *Citrullus lanatus* (Gill *et al.,* 2013). Increased GR activity has been widely observed in plant species like *T. aestivum* (Hasanuzzaman *et al.,* 2012), *Z. mays* (Kumar *et al.,* 2012), *Cucumis sativus* (Dai *et al.,* 2012), *N. tabacum* (Tan *et al.,* 2011) and *Phaseolus aureus* (Kumar *et al.,* 2011) under high temperature (HT) stress. Thus, the upregulation in the expression of GR is postulated to play an important role in plant protection against various forms of abiotic stresses (Trivedi *et al.,* 2013; Gullner *et al.,* 2001; Reisinger *et al.,* 2008).

The overexpression of enzymes of the ascorbate-glutathione (AsA-GSH) pathway has been demonstrated to confer abiotic stress tolerance in plants by reducing the stress-induced cellular reactive oxygen species (ROS). The work has been critically reviewed by Hasanuzzaman *et al.,* (2019). It has been observed that in *Vigna radiata* (Nahar *et al.*, 2016) and *Solanum lycoperisum* (Sabeeha *et al.*, 2022), the upregulation of AsA-GSH cycle components leads to saline stress and heavy metal (mercury) stress tolerance, respectively. The overexpression of AsA-GSH enzymes from *Pennisetum glaucoma* in *S. lycopersicum* has been reported to be responsible for the reduced accumulation of malondialdehyde and H_2O_2 (Raja *et al.*, 2022). The impairment of AsA-GSH cycle enzymes has been demonstrated to increase saline stress sensitivity in *A. thaliana* (Huang *et al.*, 2005). Further, the overexpression of *Malpighia glabra* monodehydroascorbate reductase (MDHAR) leads to saline stress tolerance in *Nicotiana tabacum* (Eltelib *et al.*, 2012). The transgenic *O. sativa* plants overexpressing MDHAR from *Acanthus ebracteatus* were found to demonstrate enhanced saline tolerance (Sultana *et al.*, 2012). The overexpression of MDHAR and dehydroascorbate reductase (DHAR) in *N. tabacum* (Eltayeb *et al.*, 2007) and *A. thaliana* (Ushimaru *et al.*, 2006) have been demonstrated to increase saline stress tolerance. The overexpression of APX has been demonstrated to reduce the toxic effects of stress-induced H_2O_2 and enhance salinity tolerance in *N. tabacum* (Badawi *et al.*, 2004). Similarly, the overexpression of APX in *N. tabacum* has been demonstrated to relieve it from high and low-temperature stress (Yabuta *et al.*, 2002).

It has also been suggested that different GR isoforms can be stimulated by varied environmental signals and can have different functional manifestations in the response to stress in plants (Stevens *et al.,* 1997). This further supports our analysis that a peroxisomal localized GR could have a more pronounced effect in imparting ROS scavenging properties to plants and hence providing better abiotic stress tolerance. It is also pertinent to mention here that ample literature is available about chloroplastic and cytosolic forms while no work has yet been reported on peroxisomal GR. This would be the first report of a full-length GR to be localized in peroxisome from a monocot plant and its involvement in abiotic stress tolerance. Since both peroxisome and GR are the key entities in cellular ROS homeostasis, a peroxisome localized GR would be of much significance in contributing to the abiotic stress tolerance in plants. The *in silico* analysis also revealed that the peroxisomal localization of GR could be a universal feature among angiosperms. Further, we also reported that the GR was induced by multiple abiotic stress conditions, suggesting that this could also be effective in providing stress tolerance under stress combination or stress matrix situations as well, however, further studies need to be done in this regard.

CONCLUSION

In the nutshell, we reported a peroxisome localized GR from the monocot model plant *Oryza sativa*. The *in silico*

analysis also revealed that the peroxisomal localization of GR could be a common feature amongst angiosperms. The peroxisome localized GR was also found to be induced by multiple abiotic stress conditions. The role of GR in ROS homeostasis combined with its peroxisome localization and induction by multiple abiotic stress conditions suggests that it could be a prime candidate for the development of tolerance against abiotic stress combinations.

Declarations

Conflict of interests. The authors confirm that there are no conflicts of interest.

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