Biochemical and Biophysical Research Communications 458 (2015) 536–542

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

In vitro and *in vivo* evidence for oxalate oxidase activity of a germin-like protein from azalea

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ARTICLE INFO

Article history:
Received 23 January 2015
Available online 10 February 2015

Keywords:
Cupin
Germin-like protein
Extracellular matrix protein
Oxalate oxidase
Superoxide dismutase
Hydrogen peroxide

ABSTRACT

Germins and germin-like proteins (GLPs) comprise large families of extracellular plant glycoproteins that are structurally similar, yet they have been reported to have distinct biochemical activities: oxalate oxidase and superoxide dismutase activities, respectively. We expressed an azalea GLP (RmGLP2) in cultured cells of tobacco, and determined that the extracellular protein fraction and the recombinant RmGLP2 protein purified from these cells catalyzed the oxidation of oxalate. Notably, this activity is purportedly restricted to germin and has not been demonstrated for a GLP. Although the specific activity of the purified RmGLP2 protein was low compared with that of a previously characterized barley germin/oxalate oxidase, tobacco cells expressing RmGLP2 exhibited significantly reduced oxalate levels. Thus, RmGLP2 represents the first reported GLP with oxalate oxidase activity.

1. Introduction

Germins and the closely related germin-like proteins (GLPs) constitute a superfamily of plant glycoproteins that are mainly found in the apoplast or extracellular matrix [1–3]. While the occurrence of germins is restricted to monocotyledonous true cereals, it appears that GLPs are ubiquitous in land plants, spanning a taxonomic range from bryophytes to angiosperms, and generally encoded by multigene families [4–6]. Phylogenetically, both germins and GLPs belong to the cupins, a large and functionally diverse superfamily of proteins that have a conserved barrel-like architecture, typically consisting of antiparallel β-sheets [7]. Structural elements that are characteristic of germins and GLPs include an amino-terminal secretory signal peptide, two histidine-containing motifs that form a catalytic β-barrel core involved in manganese-cofactor binding, and a carboxyl-terminal α-helix domain. Crystallographic studies have shown barley germin, and so by inference GLP, to be a tightly packed homohexamer comprising a trimer of dimers that is stabilized by hydrophobic interactions within the complex [8]. It has been proposed that this quaternary structure mostly explains their exceptional resistance to heating, extreme pH, proteolysis and treatment with sodium dodecyl sulfate (SDS) [9–11].

Germin family genes show differences in expression and regulation with respect to tissue distribution, developmental processes and stress responses, suggesting a wide range of the physiological roles [3]. Although the functions of most of the available germin-related sequences are unknown, individual members have been shown to exhibit different biochemical activities, including oxalate oxidase (OXO) [12,13], manganese superoxide dismutase (SOD) [8,10,14], ADP-glucose pyrophosphatase/phosphodiesterase [15] and serine protease inhibitor [16] activities, while others have been suggested to act as receptors or structural proteins [17–19]. Among these reported activities, notable enzymes are OXO and SOD, both of which produce H_2O_2, a mediator of oxidative stress and cellular signaling, which has led to the suggestion that they play roles in cell wall modification and responses to biotic and abiotic stresses [2,3]. To date, true germins, which are found in cereal monocots, have exclusively been associated with OXO activity, while GLPs from various species have been shown to have activity of SOD but not of OXO [3,6], with the only exception being a barley germin that has been reported to have both activities [8].

Previously, we reported the cloning of two GLP genes (*RmGLP1* and *RmGLP2*) from a woody azalea (*Rhododendron mucronatum* G. Don) [20], a species that is often used as a roadside shrub for urban greening and possible remediation of vehicular exhaust pollution. We also determined that the expression of both genes is activated

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http://dx.doi.org/10.1016/j.bbrc.2015.02.002
0006-291X/

by exposure to air-polluting nitrogen oxides [20]. Moreover, when produced as recombinant proteins in cultured tobacco cells, RmGLP2 was found to be expressed as a salt solution-extractable apoplastic protein with SOD activity [20]. Here we report the purification and further characterization of recombinant RmGLP2, and the unexpected discovery that this particular SOD-GLP also functions as OXO *in vitro* and in living cells. To our knowledge, this is the first report of a GLP with OXO activity.

2. Materials and methods

2.1. Plant cells and culture conditions

A tobacco (*Nicotiana tabacum* L.) suspension-cultured cell line Bright Yellow-2 (BY-2) was propagated in a modified liquid Linsmaier and Skoog medium (pH 5.6). The suspension was subcultured at 7-day intervals in 300-ml Erlenmeyer flasks containing 70 ml of the medium at a constant temperature of 26 °C under continuous dark conditions on an orbital shaker at 130 rpm. BY-2 calli were maintained on the same medium that was solidified with 0.4% (w/v) Gellan Gum (Wako) and subcultured every month.

2.2. Recombinant production of RmGLP2 in tobacco and bacterial cells

Construction of the expression vector to generate a precursor form of RmGLP2 with the putative signal peptide and a carboxyl-terminal hexahistidine (His_6) tag and the stable transformation of BY-2 cells have been described previously [20]. The mature form of the His_6-tagged RmGLP2 lacking the putative signal peptide was produced in *Escherichia coli* as previously described [20].

2.3. Preparation of extracellular matrix proteins

Extracellular matrix proteins were prepared from 3-week old callus by shaking in an equal volume of 1 M NaCl for 30 min without mechanical cell disruption, followed by concentration and desalting using a 10 kDa cut-off centrifugal filter (Millipore). Protein levels were quantified using a protein assay kit (Bio-Rad) with bovine serum albumin (BSA) as a standard.

2.4. Purification and on-column refolding of recombinant proteins

His_6-tagged recombinant proteins were obtained by single-step on-column affinity refolding purification using a nickel-chelating column as previously described [21] with minor modifications, as detailed in Supplementary Methods.

2.5. Enzyme assay

OXO activity was assessed by in-gel activity staining and in-solution spectrophotometric assays that visualized oxalate-dependent H_2O_2 production through horseradish peroxidase (HRP)-catalyzed oxidation of dye substrates in the presence of ethanol [22]. For in-gel assays, after the native proteins were separated by non-reducing 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the gels were immersed for 1–3 h at 25 °C in 60% (v/v) ethanol containing 40 mM succinic acid/NaOH (pH 3.8), 2 mM oxalic acid, 5 U/ml HRP and 0.5 mg/ml 4-chloro-1-naphthol (4CN) as a chromogenic reagent. The spectrophotometric assay was conducted at 37 °C in the same reaction, except that instead of using 4CN, H_2O_2 production was evaluated by monitoring absorbance at 555 nm due to the formation of a dye from HRP-mediated coupling between *N*,*N*-dimethylaniline (0.2 μl/ml) and 4-aminoantipyrine (80 μg/ml). In both assays, oxalic acid was omitted in control experiments and glycolic acid was applied as an inhibitor. Negative staining of SOD activity was performed as previously described [20].

2.6. Western and lectin blot analyses

Crude and purified proteins were loaded on SDS-PAGE gels in their native state or following heat-denaturation and reduction in the presence of SDS and β-mercaptoethanol (β-ME), and analyzed by Western blotting, using the primary rabbit antibody raised against recombinant RmGLP2 [20]. The HRP-conjugated goat secondary antibody was used for detection of antigen–antibody complexes with chromogenic 4CN or chemiluminogenic luminol as a substrate. For lectin blotting, purified recombinant proteins were resolved by 12% SDS-PAGE under reducing condition and then blotted onto a polyvinylidene difluoride membrane. After washing four times in Tris-buffered saline, pH 7.5, with 0.05% (v/v) Tween 20 (TBST), the membrane was incubated overnight in 3% (w/v) BSA and then for 1 h with the mixture of eight HRP-lectin conjugates (0.1 μg/ml; J-Oil Mills, Inc.). The membrane was washed three times in TBST and then oligosaccharides were detected by luminol-dependent chemiluminescence.

2.7. Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) peptide mass fingerprinting (PMF) analysis

Purified recombinant proteins were separated by 12% SDS-PAGE under reducing conditions. After staining with Coomassie brilliant blue (CBB), the major band was excised and subjected to the PMF analysis by MALDI-TOF mass spectrometry as described in Supplementary Methods.

2.8. Oxalic acid content

Callus tissues after 3 weeks of subculture were homogenized in an equal volume of 1 M HCl, incubated at 95 °C for 30 min, and centrifuged at 14,000× *g* to obtain a clear supernatant. After the pH of the supernatant was adjusted to 3.0, total oxalic acid levels were determined using a commercially available quantification kit (R-Biopharm AG).

3. Results and discussion

OXO catalyzes the manganese-dependent oxidative decomposition of oxalate to carbon dioxide, with equimolar consumption of O_2 and production of H_2O_2. OXO activity was the first biochemical activity identified among the germin family proteins and is still associated exclusively with the true germins that are found only in monocot cereals, such as barley and wheat [12,13]. Despite structural similarities, all the GLPs examined to date have been reported to lack OXO activity, although in most cases these studies employed standard assay methods rather than a more sensitive method [22] (Supplementary Table 1, and references therein). In this study, we explored the possibility that RmGLP2 possesses OXO activity, using transgenic tobacco cells and the purified recombinant protein.

3.1. RmGLP2 over-expression in tobacco cells results in the appearance of extracellular OXO activity

We previously reported the establishment of transgenic tobacco BY-2 cell lines expressing carboxyl-terminal His_6-tagged RmGLP2 as a salt-extractable extracellular matrix protein that exhibits SOD activity [20]. These cell lines were examined for the presence of the recombinant RmGLP2 protein in the extracellular fraction of the

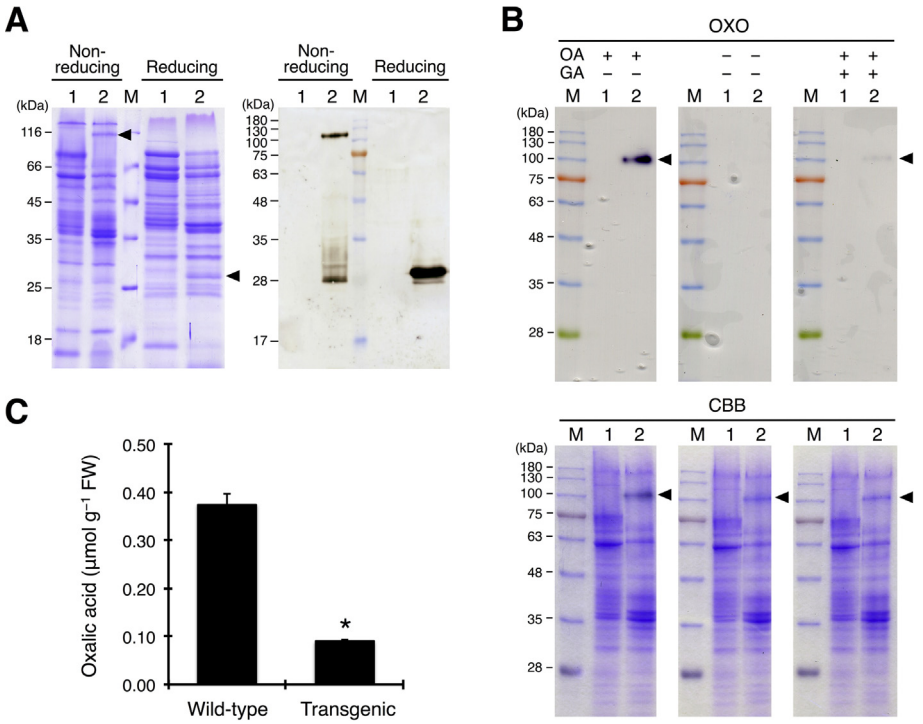

Fig. 1. Occurrence of extracellular OXO activity in transgenic tobacco cells expressing recombinant RmGLP2. (A) Detection of RmGLP2 in the extracellular fraction. Extracellular matrix proteins (5 μg) were resolved by 12% SDS-PAGE under non-reducing or reducing conditions, after which RmGLP2 was detected by CBB staining (left, indicated by arrowheads) and Western blot analysis, using anti-RmGLP2 antibodies (right). (B) OXO activity in the extracellular fraction. Extracellular matrix proteins (5 μg) were resolved by non-reducing 10% SDS-PAGE and stained for OXO activity in the presence or absence of substrate (OA, 2 mM oxalate) and inhibitor (GA, 20 mM glycolate). Below are the same gels that were stained with CBB to show the presence of RmGLP2 (indicated by arrowheads). Lane M, Dalton markers; lane 1, wild-type cells; lane 2, transgenic cells. The gel images shown are representative of at least three independent experiments. (C) Oxalic acid levels in wild-type and transgenic cells. FW, fresh weight. The mean values ± standard deviations ($n = 3$, $^*P < 0.001$ by Student's t-test) are shown.

callus tissues by SDS-PAGE. Fig. 1A shows the CBB-staining patterns and parallel Western blot results, which revealed that the recombinant RmGLP2 was clearly visible in the SDS-PAGE gels, and occurred as oligomers (ca. 120 kDa) under non-reducing conditions, consisting of 26-kDa monomer subunits as determined by fractionation under reducing conditions.

To assess the possible OXO activity of the recombinant RmGLP2 protein, we employed an ethanol-based method for detecting and measuring the activity, as this assay is considered to be the most sensitive yet developed [22]. Using in-gel activity staining, we compared oxalate-dependent H_2O_2 production from the extracellular protein fraction of the transgenic cells with that of the wild-type (WT) BY-2 cells. Under our experimental conditions, no staining signal was generated by the WT fraction after overnight incubation in the presence of oxalate. However, the extracellular fraction from the transgenic lines produced a single clear signal of OXO activity, typically in one to a few hours, which corresponded well in molecular mass to that of the native recombinant RmGLP2 oligomers, as shown by subsequent CBB staining of the same gels (Fig. 1B). This signal was totally absent from the transgenic fraction when oxalate was excluded from the assay, and significantly weakened when glycolate, a non-metabolized analog of oxalate, was included in 10-fold excess over the substrate. We note that we confirmed that glycolate did not interfere with the HRP reaction at the concentration used. Moreover, consistent with the defining characteristic of germin and GLP, the staining profile of the transgenic fraction was maintained after heating at 80 °C for 5 min (Supplementary Fig. 1). These results strongly suggest that recombinant RmGLP2 can catalyze oxalate-dependent H_2O_2 generation *in vitro*.

3.2. Heterologous RmGLP2 over-expression reduces oxalate levels in tobacco cells

To obtain further evidence that recombinant RmGLP2 can act as an OXO, we measured total oxalic acid content in the WT and transgenic calli. Since oxalate is often associated with the plant cell wall [23], we predicted that its level would be lower in the transgenic cells than in the WT cells if RmGLP2 exhibited OXO activity *in vivo*. Indeed, coincident with the apoplastic accumulation of RmGLP2 (Fig. 1A), transgenic cells had oxalate levels that were one fourth of those in the WT cells (Fig. 1C). The results thus suggest that recombinant RmGLP2 has the capacity to decompose oxalate *in vivo*.

3.3. Recombinant RmGLP2 is glycosylated and possesses low, but significant, OXO activity

We attempted to isolate the His_6-tagged recombinant proteins directly from the extracellular fraction of the transgenic cells by affinity purification using nickel column chromatography; however, this approach was not successful. This led us to take a reconstitution approach in which the extracellular matrix proteins were denatured using urea and β-ME, and the resulting dissociated polypeptides were bound to the nickel-immobilized resin for on-column refolding. By stepwise removal of the denaturing and reducing reagents during column washing, this refolding protocol yielded a protein of the predicted molecular masses by non-reducing and reducing SDS-PAGE (Fig. 2A). Western blot analysis showed that the denatured polypeptides consisted of two closely migrating bands, which most likely reflected a characteristic

doublet of *N*-glycosylated and non-glycosylated forms that have previously been reported for GLPs [10,14]. We verified this possibility by lectin blotting using the bacterially expressed recombinant protein as a non-glycosylated control, which identified only the major, more slowly migrating, polypeptide as being glycosylated (Fig. 2B). These results demonstrated that RmGLP2 occurs mostly in a glycosylated form following heterologous expression in tobacco cells.

MALDI-TOF PMF analysis of the major polypeptide after in-gel trypsin digest resulted in the identification of 13 peptides, which collectively covered 63.5% of the theoretical 222-residue precursor sequence of RmGLP2, including the putative signal peptide and the carboxyl-terminal His_6 tag (Fig. 2C, Supplementary Table 2). Two non-identified regions correspond to the amino-terminal 24 residues and an internal 23-residue stretch that contains two potential *N*-glycosylation sites (Fig. 2C, inset). The amino-terminus of the purified protein exactly matched that of the previously predicted mature protein [20], establishing that the first 24 residues constitute the signal peptide for extracellular secretion. Failure to identify the internal region probably reflects the presence of *N*-glycosylation, as shown above, possibly at Asn-70 (the 46th residue of the mature protein), based on the reported position of the glycosylated residue of a barley germin [8].

The in-gel assay demonstrated the SOD activity of the purified protein (Fig. 3A), confirming that recombinant RmGLP2 was functionally reconstituted during on-column affinity refolding purification. The assay also detected the OXO activity that was absolutely dependent on oxalate and sensitive to inhibition by the substrate analog glycolate (Fig. 3A), thus reproducing the previous results with the extracellular matrix proteins (Fig. 1B). These results indicate that the observed OXO activity of the purified protein was not an artifact resulting from the reconstitution of the dissociated polypeptides during the purification process, but rather was intrinsic to the properties of RmGLP2. The OXO activity of the purified RmGLP2 was also evidenced by an in-solution assay that gave results consistent with those obtained by the in-gel assay (Fig. 3B). However, taking into account the equimolar O_2 consumption and H_2O_2 production in the OXO reaction, the specific activity of the purified RmGLP2 (201 ± 31.9 nmol H_2O_2 produced/mg/min, Fig. 3B)

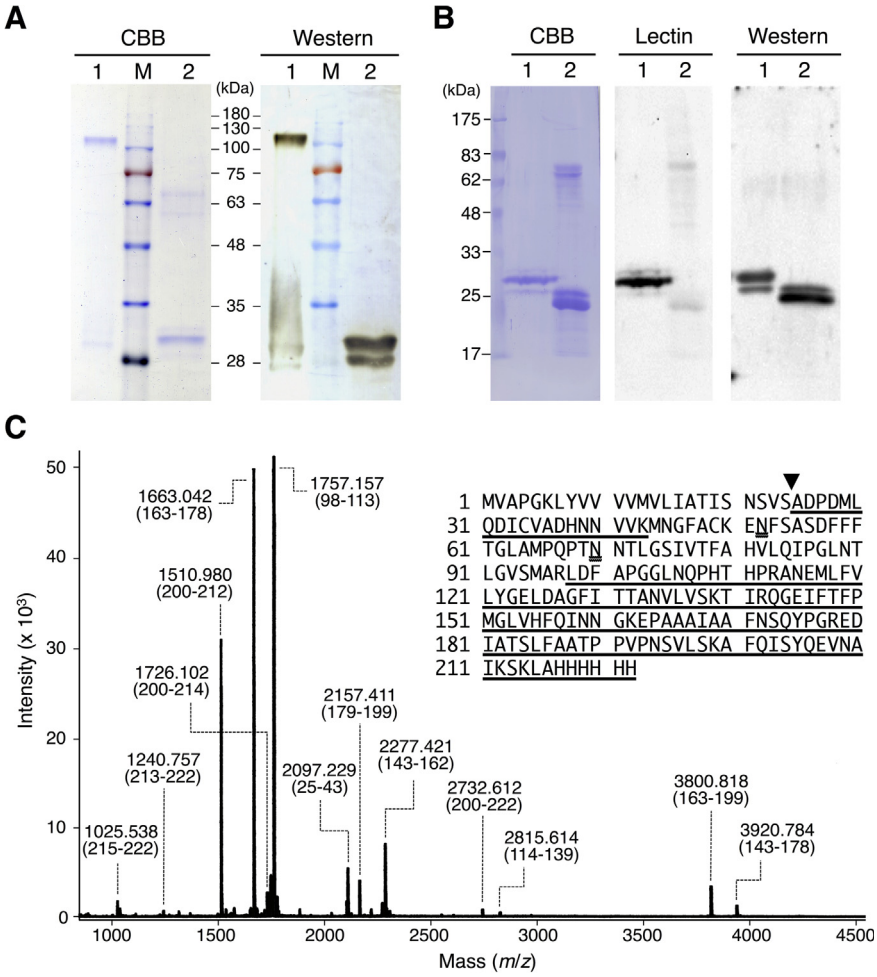

Fig. 2. Purification and identification of recombinant RmGLP2 from tobacco cells. (A) CBB staining and Western blot analysis of the purified recombinant proteins (1 μg) after 10% SDS-PAGE under non-reducing (lane 1) and reducing conditions (lane 2). Lane M, Dalton markers. (B) Lectin and Western blot analysis of recombinant RmGLP2 from tobacco (lane 1) and *E. coli* (lane 2). Nickel-affinity purified proteins (5 μg) were subjected to 12% SDS-PAGE under reducing conditions, blotted onto the membranes, and probed with a mixture of eight lectin-HRP conjugates or an anti-RmGLP2 antibody. Lectin blots were stained with CBB to verify protein transfer. (C) MALDI-TOF PMF spectrum of the purified protein. Signals that matched the theoretical peptide masses of His_6-tagged RmGLP2 are labeled with *m*/*z* values and corresponding residue numbers in parentheses (see also Supplementary Table 2). The inset shows the full RmGLP2 sequence where the mass-matched peptides are underlined and the processing site of the signal peptide is marked by an arrowhead. Putative *N*-glycosylation residues are double-underlined.

was about 50-fold lower than that reported for the recombinant barley germin OXO, also known as HvGER1a (10.26 ± 0.85 μmol O_2 consumed/mg/min) [24]. As discussed below, this could be partly attributable to a single residue difference in the active site between the two proteins. Nevertheless, these results indicate that RmGLP2 possesses significant, albeit low, OXO activity *in vitro*; a conclusion that is supported by the *in vivo* evidence of the reduced oxalate levels in tobacco cells expressing RmGLP2 (Fig. 1C). We note that a sugarbeet GLP has been suggested to possess OXO activity, although the protein was not purified and the specific activity was unconfirmed [25].

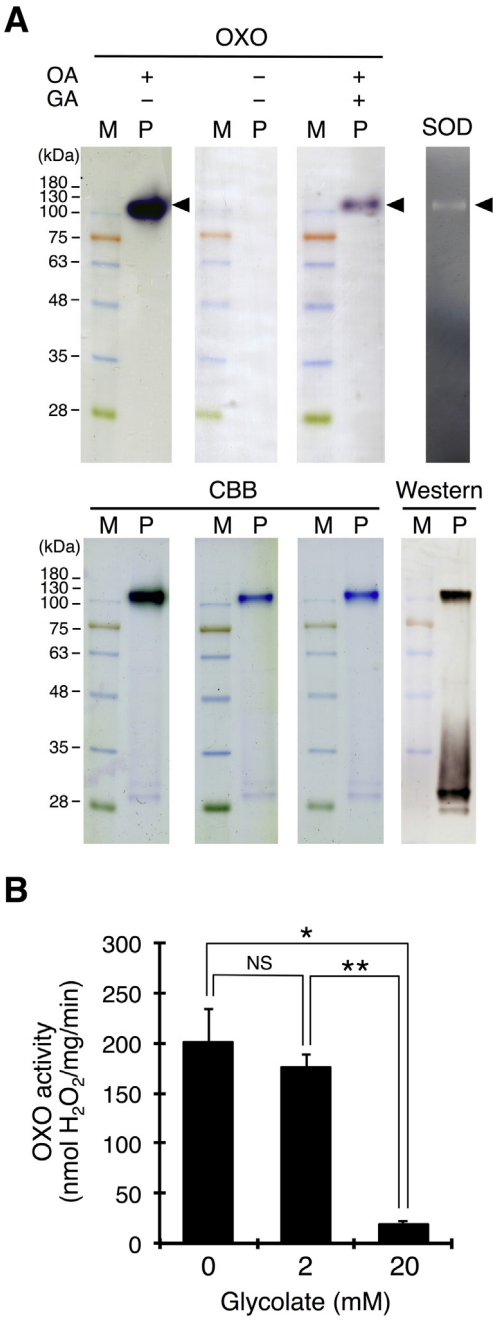

Fig. 3. Enzyme activities of recombinant RmGLP2. (A) Purified proteins (0.5 μg, lane P) were resolved by non-reducing 10% SDS-PAGE for staining SOD activity and OXO activity in the presence or absence of substrate (OA, 2 mM oxalate) and inhibitor (GA, 20 mM glycolate). Visualized activities are indicated by arrowheads. Shown below are images of the same gels stained with CBB and the parallel Western blots. Lane M, Dalton markers. The gel images are representative of at least three independent experiments. (B) Specific OXO activities and the effects of glycolate. The in-solution enzyme assay was performed as described in Materials and Methods, with 2 mM oxalate and the indicated concentrations of glycolate. The mean values ± standard deviations ($n = 3$, $^{*}P < 0.01$ and $^{**}P < 0.005$ by Student's *t*-test) are shown. NS, non-significant ($P > 0.05$).

3.4. GLPs do not have an aspargine residue that may be crucial for OXO activity

The catalytic mechanism of oxalate degradation by OXO has been proposed through elucidation of the crystal structures of the native and recombinant forms of the barley germin protein HvGER1a [8,24]. These stereoscopic studies identified two groups of a total of seven amino acid residues involved in the catalysis: Asn-75, Asn-85 and Gln-139 that bind the substrates and reaction intermediates, and His-88, His-90, Glu-95 and His-137 that coordinate the manganese-cofactor at the reaction center. When compared with the sequence of this barley germin OXO, RmGLP2 shows conservation of all seven residues, other than Asn-75, which is Ala at the equivalent position (Fig. 4A). Importantly, site-directed mutagenesis of HvGER1a to substitute Asn-75 for an Ala significantly decreased its OXO activity by 43-fold [24], and the resultant activity of the mutated protein (0.24 ± 0.02 μmol O_2 consumed/mg/min) was roughly comparable to that of the purified RmGLP2 (Fig. 3B). We therefore propose that this single residue difference might be, at least in part, responsible for the weak OXO activity of RmGLP2, although the involvement of other residues cannot be excluded.

To examine this inference further, we compared 74 germin family sequences covering a broad phylogenetic range (Supplementary Table 3), by focusing on the aforementioned seven residues. These sequences, comprising 14 germin and 60 GLP members, were used in a recent phylogenetic analysis [6], where they were classified into six representative subfamilies (GER1 to GER6) and two unnamed clades. As illustrated in Fig. 4B, most of the seven residues are conserved across the entire germin family. However, Asn-75 is strictly unique to the GER1 subfamily, which contains true cereal germins with experimentally demonstrated OXO activity [12,13]. In contrast, none of the 60 GLP sequences analyzed, that constitute all the other subfamilies and clades, has Asn or a similar residue at the corresponding position (i.e. Ala, Val or Ser). The apparently exclusive conservation of Asn-75 in the GER1 subfamily supports its importance in germin OXO activity, as reported by mutation analysis of HvGER1a [24]. Since molecular phylogenetic analyses have confidently shown that the GER1 subfamily (true germin OXO clade) branches from the ancestral GLP [4–7], the mutation to create the Asn residue at the active site may be considered as an important event in the evolution of germin OXO proteins. Conversely, the total absence of such a potentially critical residue in any other phylogenetic groups seems to explain, at least in part, why GLPs generally exhibit low (this study), or no OXO activity (Supplementary Table 1).

In summary, the present report provides the first demonstration of a GLP, or dicotyledonous germin family protein, with detectable OXO activity, pointing to the need to revisit and examine the enzyme activities of GLPs using a sensitive assay protocol [22]. Since the catalytic properties of the vast majority of GLP sequences have not been examined (by the end of 2014, more than 3700 nucleotide sequences with the annotation “germin” or “germin-like” were present in the GenBank database), it remains to be seen whether OXO activity represents an unappreciated function of these functionally diverse protein families.

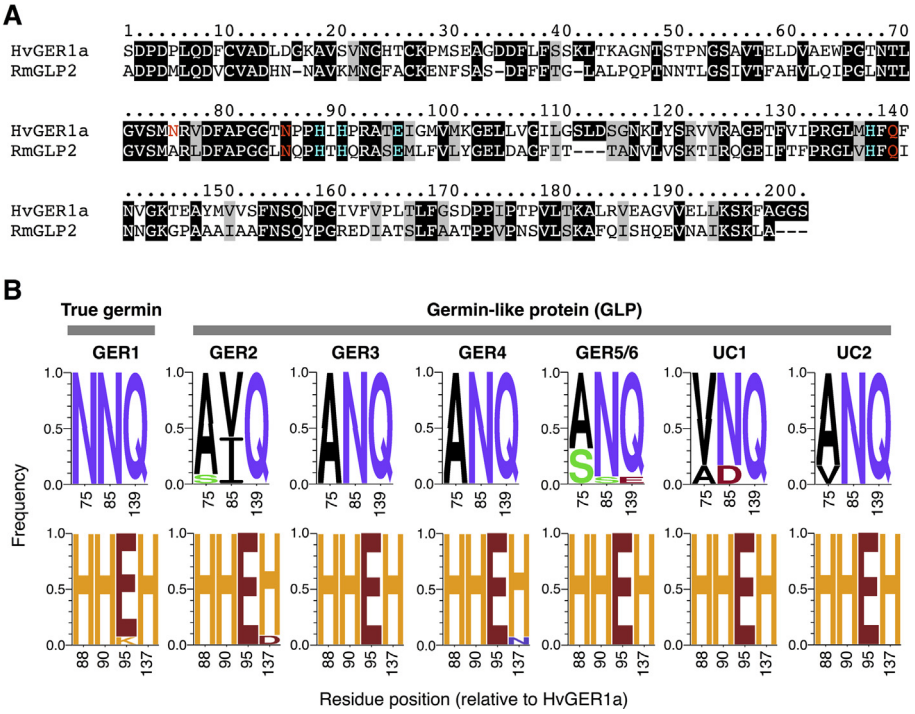

Fig. 4. Sequence analysis of germin family proteins in relation to OXO activity. (A) Comparison of amino acid sequences of RmGLP2 and barley germin OXO (HvGER1a). Sequences were aligned using ClustalW 2.1 (http://clustalw.ddbj.nig.ac.jp/) and numbered according to the barley sequence. Amino acid identities are indicated by black boxes and similarities by gray boxes using Boxshade 3.3.1 (http://mobyle.pasteur.fr/cgi-bin/portal.py#forms::boxshade). The residues involved in substrate binding and those coordinating the manganese-cofactor are highlighted in orange and cyan, respectively. (B) Amino acid frequencies of germin family proteins at the positions equivalent to Asn-75, Asn-85 and Gln-139 for substrate binding, and to His-88, His-90, Glu-95 and His-137 for coordinating the manganese-cofactor, in the barley germin HvGER1a sequence. A total of 74 sequences from 18 plant species were analyzed (Supplementary Table 3). These sequences are classified into 6 representative subfamilies (GER1 to GER6) and 2 unnamed clades (UC1 and UC2) in the phylogenetic tree, as previously reported [6]. The numbers of sequences analyzed are: GER1, 14 (including HvGER1a); GER2, 12; GER3, 6; GER4, 14; GER5/6, 16 (including RmGLP2); UC1, 6; UC2, 6. For each subfamily and clade, sequences were aligned by ClustalW 2.1 and analyzed for the frequency of amino acid residues at the specified positions using WebLogo 3.4 (http://weblogo.threeplusone.com/). The residues are colored based on their physicochemical properties: aliphatic/hydrophobic, black; basic, orange; acidic, brown; polar, slate-blue; hydrophilic, green.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research on Innovative Areas (grant nos. 23119514 and 25119717 to A.S.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.002.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.002.

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