Molecular characterization of atmospheric NO_2-responsive germin-like proteins in azalea leaves

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Abstract

Atmospheric nitrogen dioxide (NO_2) is an environmental oxidant that is removed through direct uptake by foliage, but plant responses to this highly reactive gas are not well understood at the molecular level. From NO_2-exposed leaves of a woody azalea (*Rhododendron mucronatum*), we cloned two cDNAs (*RmGLP1* and *RmGLP2*) for germin-like proteins (GLPs), a group of ubiquitous plant proteins that have been implicated in various plant physiological and developmental processes. Quantitative analysis of mRNA expression, together with immunoblotting data, showed that foliar exposure to NO_2 caused a robust induction of these *GLP*-encoding genes. When produced in tobacco cell culture, recombinant RmGLP2 was secreted into the apoplast, where it exhibited superoxide dismutase activity. *RmGLP1* and *RmGLP2* represent the first examples of plant genes that are responsive to airborne NO_2. These enzymes might have a potential role in extracellular defense mechanisms through attenuation of interactions between reactive nitrogen and oxygen species.

Key words: Germin-like protein; GLP; nitrogen dioxide; nitric oxide; reactive nitrogen species; superoxide dismutase; reactive oxygen species

Introduction

Nitrogen dioxide (NO_2), together with nitrogen monoxide (or nitric oxide: NO), constitutes a major type of air pollutant which influences atmospheric chemistry and aerosol formation and is of critical concern to the global environment. Because of its reactive nature, atmospheric NO_2 is generally harmful to organisms that are exposed to this gaseous free radical [1]. Although it is still contentious, plant vegetation is considered to function as a sink for removing environmental air NO_2 through foliar uptake and subsequent assimilation into amino acids after spontaneous breakdown of NO_2 into inert nitrate and nitrite [2,3].

Rhododendron mucronatum, a broadleaf evergreen azalea cultivar, is a landscaping shrub widely used in Japan as roadside vegetation. As a target breeding plant for the practical removal of air pollution and as a model plant for investigation of plant defense responses to NO_2, we examined this woody plant for its ability to uptake and assimilate NO_2 [4,5]. Attempts were also made to characterize the foliar proteins that respond to hazardous levels of NO_2 fumigation [6]. An early preliminary analysis suggested the existence of potential NO_2-responsive proteins which included a homolog to germin-like protein (GLP) [6]. Germin and GLP are members of a large and diverse family of plant proteins that are phylogenetically divided into five subgroups [7]. These proteins consist of extremely stable oligomers containing a core ß–barrel and are expressed as extracellular glycoproteins frequently associated with the cell wall [8]. Expression of the germin/GLP multigene family appears to be differentially regulated during development and by various environmental signals [8]. Only a limited number of the members of this family have been characterized as possessing certain biochemical functions, most notably the ability to generate H_2O_2, as occurs with oxalate oxidase (OxO) [9] and superoxide dismutase (SOD) [7]. Although their physiological role is not clearly understood, these proteins have been suggested to function in cell wall

modification in order to control plant growth and biotic stress responses for defense against pathogens [8,10].

Here we report the cloning of two cDNAs encoding GLPs (RmGLP1 and RmGLP2) from azalea leaves fumigated with airborne NO_2. The expression of these genes was significantly enhanced in response to foliar exposure to this reactive nitrogen gas. Transgenic production of RmGLP2 in tobacco cell culture showed RmGLP2 to predominantly be an apoplastic protein with SOD activity. Based on these results, we discuss plausible putative functions for these inducible GLPs during NO_2 exposure of the azalea leaves.

Materials and methods

Plant material and exposure to NO_2 gas. Azalea (*Rhododendron mucronatum* G. Don) plants were selected for phenotypic homogeneity consisting of a height of approximately 30 cm and were purchased from a local nursery. These plants were immediately replanted into pots containing artificial mould soil and grown for 2 weeks in a greenhouse. Foliar exposure to NO_2 was performed essentially as previously described [4]. Potted plants were transferred to a fumigation chamber where they were allowed to acclimate for 8 h to the chamber conditions of 70 μmol photons m^{-2} s^{-1}, 22.0 ± 0.3°C, 70 ± 4% relative humidity, and 0.03% to 0.04% CO_2. Plants were then exposed for up to 8 h to NO_2 at 4.0 ± 0.1 ppm during daylight hours (9 AM to 5 PM) when stomata were fully open. At 0, 4, and 8 h following the start of the exposure period, ten leaves from the shoot apex were collected from three plants per treatment. Ambient air (below 20 ppb NO_2) was used for control experiments.

cDNA cloning. Polyadenylated RNA from NO_2-exposed leaves (8 h) was reverse-transcribed using oligo(dT) primers, and the resulting cDNA was used as template for PCR with two degenerate primers that were designed for the conserved amino-terminal and

central regions of plant germins and GLPs [8]. The sense primer was 5'-GA(T/C)TT(T/C)TA(T/C)GTIGCIGA(T/C)CC-3', where I indicates inosine, and the antisense primer was 5'-AC(C/T)TC(A/G/T)G(A/T)(A/G)GC(A/T)C(C/G)(A/T)GG(A/G)TG-3, based on evaluation of 12 deduced amino acid sequences of Arabidopsis GLPs [11]. PCR was initiated at 95°C for 5 min, then subjected to 30 cycles of 95°C for 1 min, 40°C for 2 min and 72°C for 2 min, and completed by a final extension at 72°C for 5 min. After sequencing the resultant PCR fragments, full-length cDNAs were generated by rapid amplification of cDNA ends (RACE) using the 5'/3'-Full RACE Core Sets (Takara Bio Inc.) with gene-specific primers inferred from initially amplified products. RACE PCR conditions were the same as described above, except that the annealing temperature was 50°C. A partial cDNA for an azalea ß-tubulin was obtained as described in the Supplementary Information.

Reverse transcription-quantitative competitive PCR (RT-qcPCR). Total RNA from various leaf samples was used for oligo(dT)-primed cDNA synthesis. Initially, the ß-tubulin cDNA copy number was determined in each cDNA preparation by competitive PCR to check the integrity of RNA and to normalize the sample-to-sample variations in the original amount of RNA, and then quantification of *RmGLP1* and *RmGLP2* cDNAs followed. Known concentrations of competitor DNA, generated for each target using a Competitive DNA Construction kit (Takara Bio Inc.), were spiked into constant amounts of cDNA, after which PCR was performed as follows: 95ºC for 5 min, followed by 30 cycles of 95ºC for 1 min, 60ºC for 2 min and 72ºC for 1 min, with a final 5-min extension at 72ºC. Amplified products were separated in a 1.5% agarose gel, stained with ethidium bromide, and the fluorescent intensities of the appropriate bands were quantified using densitometry with the Gel Doc™ 2000 imaging system (Bio-Rad Laboratories) and Quantity One software (PDI, Inc.). The copy number of a target cDNA in each PCR was determined by comparing the specific

intensity with that of the competitor. The tubulin-specific primers were 5’-CGCTGCAGATCCTCGTCATGGTCGCTACC-3’ (sense) and 5’-CCAGTGTACCAATGCAAGAAAGCCTTCCTG-3’ (antisense). For *RmGLP1* and *RmGLP2*, an aliquot of each cDNA sample containing 5 x 10^4 copies of the tubulin cDNA was used in PCR as described above with the gene-specific primers, 5'-GTGACTCAAGCCTTTGTGGAGCAAG-3' (sense) and 5'-CCTCGCGGCCCGGATCTTGGCTGCCGAATC-3' (antisense) for *RmGLP1* cDNA, and 5'-GTGACTTTTGCTCATGTGCTGCAAA-3' (sense) and 5'-CCTCGCGGCCCGGATACTGGCTGTTGAACG-3' (antisense) for *RmGLP2* cDNA.

Antibody production. Detailed descriptions of the bacterial expression of recombinant proteins and antibody production are presented in the Supplementary Information.

Transformation of tobacco cells. In order to express RmGLP2 in cultured tobacco cells (*Nicotiana tabacum* L. cell line Bright Yellow-2 [BY-2]), the entire open reading frame with a hexahistidine (His_6)-coding sequence at its 3’end was amplified by PCR and inserted between the cauliflower mosaic virus 35S promoter and the *nos* terminator of the binary vector pIG121-Hm [12]. Detailed descriptions of plasmid construction, cell transformations, and genomic PCR for detecting the transgene are described in the Supplementary Information.

Protein extraction. Azalea leaves were extracted in pre-chilled acetone containing 10% trichloroacetic acid and 10 mM ß-mercaptoethanol (ß-ME), and then diluted with 9 volumes of acetone containing 10 mM ß-ME. After incubation at –20°C, denatured proteins were recovered by centrifugation, rinsed and lyophilized under vacuum. Lyophilized proteins were dissolved in 6 M urea and 0.2 M ß-ME, and centrifuged to remove insoluble material.

Tobacco callus was extracted in 1 M NaCl without disruption. After centrifugation, the resultant extracellular fraction was concentrated and desalted with 20 mM Na-PO_4 buffer (pH 7.4) using a Microcep™ with a 3 kDa cut-off membrane (Filtron Technology Corporation).

Whole-cell proteins were also extracted as a control. Protein concentrations were estimated using the Bio-Rad Protein Assay (Bio-Rad Laboratories).

Immunoblot analysis. Native and denatured proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 8% and 12% gels, respectively, and transferred to Immobilon™-P membranes (Millipore). Primary antibodies used were an anti-RmGLP2 rabbit antibody (this work), an anti-His_6-peroxidase mouse monoclonal antibody (Roche Diagnostics), and a rabbit antibody directed against recombinant Arabidopsis *S*-nitrosoglutathione reductase (GSNOR) [13]. For detection of RmGLP2 and endogenous GSNOR, membranes were incubated with the peroxidase-conjugated goat anti-rabbit IgG antibody (Vector Laboratories) before developing the peroxidase signals using Chemiluminescence Reagent Plus (NEN Life Science).

In-gel enzyme activity staining. Tobacco cell extracellular proteins were separated by non-reducing SDS-PAGE (8% gel) without denaturation (i.e., neither boiled nor reduced prior to electrophoresis). The activity of SOD was visualized directly in the SDS-PAGE gels by a standard negative staining method using nitroblue tetrazolium [14].

Results

Cloning and sequence analysis of Rhododendron mucronatum GLP (RmGLP)

PCR amplification of cDNAs prepared from NO_2-exposed azalea leaves resulted in an expected GLP amplicon of 269 bp. Sequencing analysis revealed the presence of two different cDNAs, for which respective full-length sequences were obtained by 5'- and 3'-RACE. The two cDNAs, designated *RmGLP1* and *RmGLP2*, encoded putative precursor polypeptides of 219 and 216 amino acids, respectively, with primary structures typical of

higher-plant germins and GLPs (Fig. 1). The deduced amino acid sequences of the two mature polypeptides share 83% identity. A BLASTP (version 2.2.17) analysis using default parameters showed that the best identity scores were to GLP2 from *Vitis vinifera* ($1e^{-66}$ and 64% identity for RmGLP1; $1e^{-68}$ and 67% identity for RmGLP2). Among biochemically characterized members of the germin/GLP family, the protein most closely related to the two RmGLP sequences was tobacco nectarin I, which has been shown to possess manganese-SOD activity [7] ($1e^{-63}$ and 64% identity for RmGLP1; $6e^{-63}$ and 63% identity for RmGLP2). In contrast, the RmGLPs exhibited much less identity (44% to 46%) to wheat gf-2.8, a true germin also known as OxO [9]. According to phylogenic classification by Carter and Thornburg [7], both RmGLP1 and RmGLP2 fell into the GLP subfamily 2 (Fig. S1). At least two members (Nectarin I and HvGER5) from subfamily 2 in the dendrogram have been shown to exhibit SOD activity [7,15], but no other biochemical activities have been reported to be associated with this subfamily.

Expression of RmGLP genes and proteins upon foliar exposure to NO_2

To examine the steady-state mRNA levels of the two *RmGLP* genes during the exposure to airborne NO_2, RT-qcPCR was performed with leaf total RNA at 0, 4, and 8 h after NO_2 exposure. For accurate quantification of expression, the copy number of individual *RmGLP* cDNAs was estimated and normalized to that of the constitutive ß–tubulin cDNA (Fig. 2A). In control plants exposed to ambient air (NO_2 < 20 ppb), the levels of both *RmGLP1* and *RmGLP2* transcripts were quite low and not significantly changed from baseline, accounting for only 1.6% to 2.7% and 1.6% to 2.5% of ß–tubulin levels, respectively. However, these two genes were dramatically upregulated upon exposure to 4.0 ppm NO_2. At 4 h after fumigation, *RmGLP1* and *RmGLP2* transcripts had accumulated to levels 6.5 and 35 times

higher than those of ß–tubulin, respectively. Exposure to NO_2 for an additional 4 h led to a further increase in transcript levels for both genes (11 and 43 times higher than ß–tubulin transcript levels, respectively, for *RmGLP1* and *RmGLP2*). No visible injury was exhibited by the leaf samples after NO_2 exposure.

We next determined whether changes in NO_2-responsive *RmGLP* gene transcription reflected the steady-state protein levels by immunoblot analysis using an anti-RmGLP2 antibody and protein isolated from the same leaf samples that had been analyzed by RT-qcPCR (Fig. 2B). This antibody was found to cross-react with recombinant RmGLP1 (Fig. S2). Consistent with the transcript accumulation, the polypeptides with a molecular mass typical of GLP subunits (20–25 kDa [8,10]) were enormously induced in the leaves 4 h after NO_2 fumigation. Levels remained high at 8 h, whereas almost no signal was visible for control leaves. These results indicate that airborne NO_2 stimulates the expression of these *GLP* genes in azalea leaves and that expression is primarily regulated at the transcriptional level.

Functional characterization of RmGLP2 expressed in tobacco cells

To investigate biochemical function of NO_2-responsive GLP, the entire coding region for the RmGLP2 precursor was constitutively expressed in tobacco BY-2 cells as a recombinant protein with a carboxyl-terminal His_6 tag (Fig. 3A). Screening by genomic PCR identified a total of nine transgenic cell lines for which the production of recombinant RmGLP2 was examined by immunoblotting (Fig. 3B and C). An anti-GSNOR antibody was simultaneously applied to detect the endogenous protein (NtGSNOR) as a loading reference. Whole-cell extracts from five transgenic cell lines clearly exhibited 26 kDa immunoreactive signals, roughly corresponding to the molecular mass of RmGLP proteins induced in NO_2-exposed

leaves (Fig. 2B). The 45 kDa NtGSNOR protein was detected in all samples, including wild-type (WT) cells. Stripping and reprobing the membrane with an anti-His_6 antibody resulted in essentially the same profile as that detected using the anti-RmGLP2 antibody (Fig. 3C), confirming the identity of the immunoreactive signal.

To examine the localization of RmGLP2 in tobacco cells, we prepared salt-extractable proteins from intact callus and probed for the recombinant protein and the endogenous GSNOR with the respective antibodies (Fig. 4A). The RmGLP2 protein was clearly recognized as a 26-kDa doublet, possibly due to post-translational glycosylation [8,10], at varying levels in all the transgenic fractions, but not in the WT sample. It is noteworthy that, at the protein level, the extracellular fraction of all transgenic samples was significantly enriched with the recombinant protein as compared with levels in the whole-cell extract (15 μg in Fig. 3C and 5 μg in Fig. 4A). The total absence of the GSNOR-derived signals in the immunoblot eliminated the possibility of intracellular protein contamination. These results demonstrated the predominant localization of recombinant RmGLP2 to the extracellular space of transgenic tobacco cells, where it was weakly bound to the cell wall by ionic interaction.

Because the quaternary structures of germin and GLP allow them to form detergent- and heat-stable oligomers [8,10] and because SOD activity has been associated with the subfamily 2 to which both RmGLP1 and RmGLP2 appear to belong (Fig. 1 and Fig. S1), we examined RmGLP2 expressed in tobacco cells for possible subunit assembly and SOD enzyme activity. When the extracellular fractions from transgenic tobacco cells were separated on SDS-PAGE under native conditions, the recombinant protein was detected by immunoblotting mainly as an apparent high-molecular-mass band of ca. 120 kDa (Fig. 4B). Negative staining for SOD activity revealed three extracellular fraction bands from both WT and transgenic cells (Fig. 4C). By comparing WT cells, however, transgenic cells had much

greater activity for the highest-molecular-mass band that indeed co-migrated with recombinant RmGLP2 in the parallel immunoblot analysis. Taken together, these results showed that transgenic cells produced RmGLP2 as active oligomers possessing SOD activity and secreted the recombinant protein efficiently into the extracellular space.

Discussion

In this study, we report the cloning and initial characterization of airborne NO_2-responsive GLPs from azalea leaves. Quantitative expression analyses of the cloned genes demonstrated the dramatic accumulation of *RmGLP1* and *RmGLP2* transcripts upon foliar NO_2 fumigation. This inducible expression was further demonstrated by the presence of a strong correlation between transcript and protein abundance. Therefore, this work represents the first example of transcriptional activation in plants by the atmospheric NO_2 gas, and suggests the potential role of this functionally ambiguous protein family in NO and reactive nitrogen biology.

The precise function of the RmGLP proteins following exposure to NO_2 remains unknown. It is possible that induction of RmGLP proteins is related to the detoxification of atmospheric NO_2 gas. However, throughout all organisms, no single enzyme has been identified that metabolizes this highly reactive form of nitrogen oxide. Rather, instead of directly detoxifying NO_2, RmGLP proteins might function in the mitigation of NO-mediated cytotoxicity. This possibility is suggested by the following considerations. Chemically, NO_2 and NO are mutually transformable. Upon interaction with water, NO_2 is decomposed to form nitrite, nitrate and NO [16,17]. As such, NO_2 in the atmosphere, once diffused through the stomata into the leaf apoplast, may partially undergo conversion to NO. Because the apoplast of plant cells is a major site of superoxide production [18], the reaction of the

resultant NO with the superoxide at a diffusion-limited rate allows for the rapid formation of highly toxic peroxynitrite, a powerful oxidant that can initiate lipid peroxidation, oxidize sulfhydryls, nitrate the aromatic residues of proteins, and mutate DNA [19]. A number of studies have indicated that the deleterious effects of NO are mediated, at least in part, by peroxynitrite. In fact, protein-tyrosine nitration has been reported in tobacco and Arabidopsis leaves under certain physiological and pathophysiological conditions that lead to enhanced NO production [20,21]. Although plant cells possess intracellular enzymes capable of scavenging peroxynitrite [21,22], no such demonstration has been reported for the extracellular counterparts. In the present study, we provide evidence that NO_2-responsive RmGLP2, when transgenically expressed in tobacco cells, is destined for the apoplast, where it constitutes a substantial portion of local SOD activity. By removing superoxide, such this apoplastic SOD activity might contribute to the prevention of extracellular generation of NO-derived highly reactive nitrogen species such as peroxynitrite.

Acknowledgments

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Figure Legends

Figure 1. Alignment of RmGLP1 and RmGLP2 with homologous proteins. Dark shading indicates exact identity and grey shading signifies similarity. The three consensus motifs (Boxes A, B, and C), which are highly conserved among the entire germin/GLP family [8], are shown with brackets. The arrowhead points to the putative cleavage site for the secretion signal peptide. The single lines above the sequence indicate putative *N*-glycosylation sites. Genbank accession numbers: RmGLP1, **AB272079**; RmGLP2, **AB272080**; *Vitis vinifera* GLP2 (VvGLP2), **DQ673106**; tobacco nectarin I, **AF132671**; wheat gf-2.8, **M63223**.

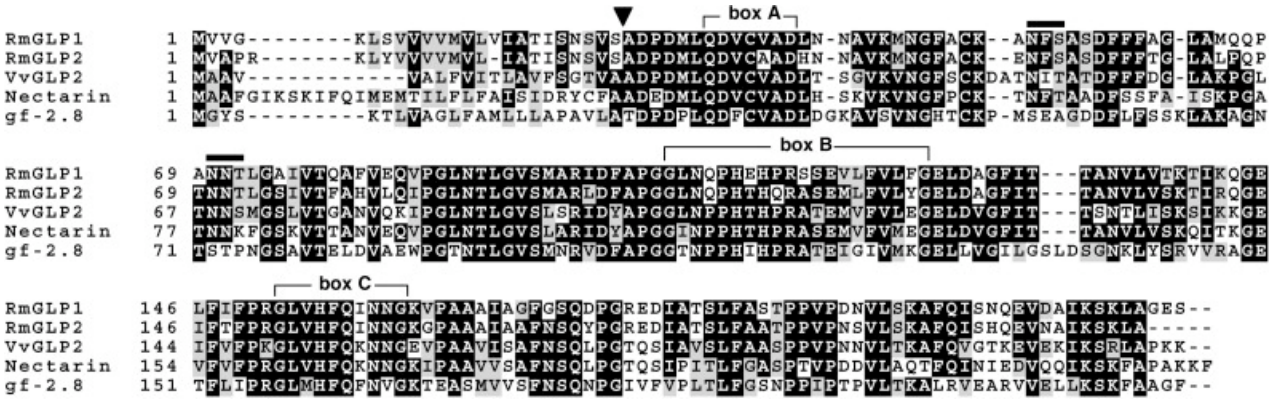
Figure 2. Induction of *RmGLP1* and *RmGLP2* expression upon foliar NO_2 exposure. Leaves were sampled at the indicated hours following the onset of foliar NO_2 exposure. (A) Quantification of *RmGLP1* and *RmGLP2* transcripts by RT-qcPCR. The copy number of each *RmGLP* transcript was normalized using that of an internal control ß-tubulin mRNA and is expressed as arbitrary units (mean ± SE, $n = 3$). Solid line, $+NO_2$ (4.0 ppm); dashed line, $–NO_2$. (B) Immunoblot analysis of leaf proteins (40 μg) probed with an anti-RmGLP2 antibody.

Figure 3. Transgenic production of recombinant RmGLP2 in tobacco BY-2 cells. (A) Schematic diagram of the transcription unit in the transformation vector (not to scale). Primer pairs used for genomic PCR are shown as arrows above the diagram. (B) Genomic PCR of tobacco cell DNA co-amplifying the *RmGLP2* transgene and the endogenous *NtNii1* gene. Lane M, 100-bp DNA ladder marker; lane N, no template; lane U, untransformed cell; lanes 1-9, independently obtained transformed cells. (C) Immunoblot analysis of whole cell extracts (15 μg protein) from transgenic tobacco cells, probed with anti-RmGLP2 and

anti-GSNOR antibodies (upper). The same blot was reprobed with anti-His_6 antibody after stripping (lower).

Figure 4. Extracellular localization, oligomerization, and enzyme activity of recombinant RmGLP2. (A) Immunoblot analysis of salt-extractable extracellular fractions (5 μg protein) from tobacco cells. The blot was prepared and probed as in Fig. 3C. (B) Immunoblot analysis of recombinant RmGLP2 after non-denaturing SDS-PAGE. Extracellular fractions (3 μg, the same as lane 1 of Fig. 4A) were subjected to analysis without (lane 1) or with (lane 2) prior heat denaturation under reducing conditions. (C) In-gel staining for SOD activity of recombinant RmGLP2. Following non-denaturing SDS-PAGE of extracellular fractions (10 μg protein), SOD activity was visualized by a standard negative staining method (left, marked by arrowheads). A gel duplicate was immunoblotted to determine the migration of recombinant RmGLP2 (right). Lane 1, untransformed cells; lane 2, transgenic cells.

Fig. 1 (Kondo et al.)



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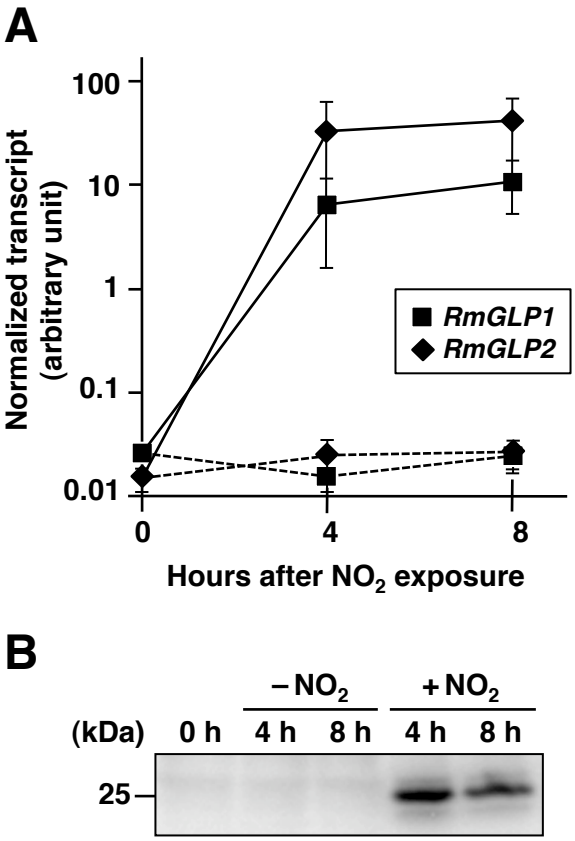
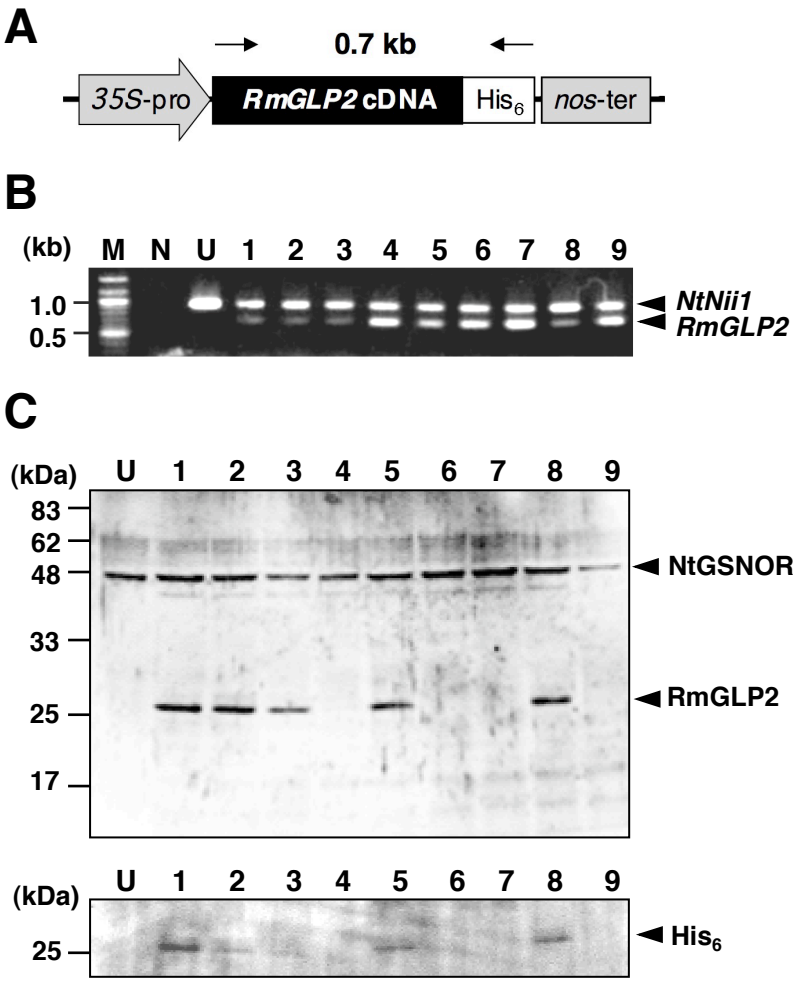


Fig. 3 (Kondo et al.)



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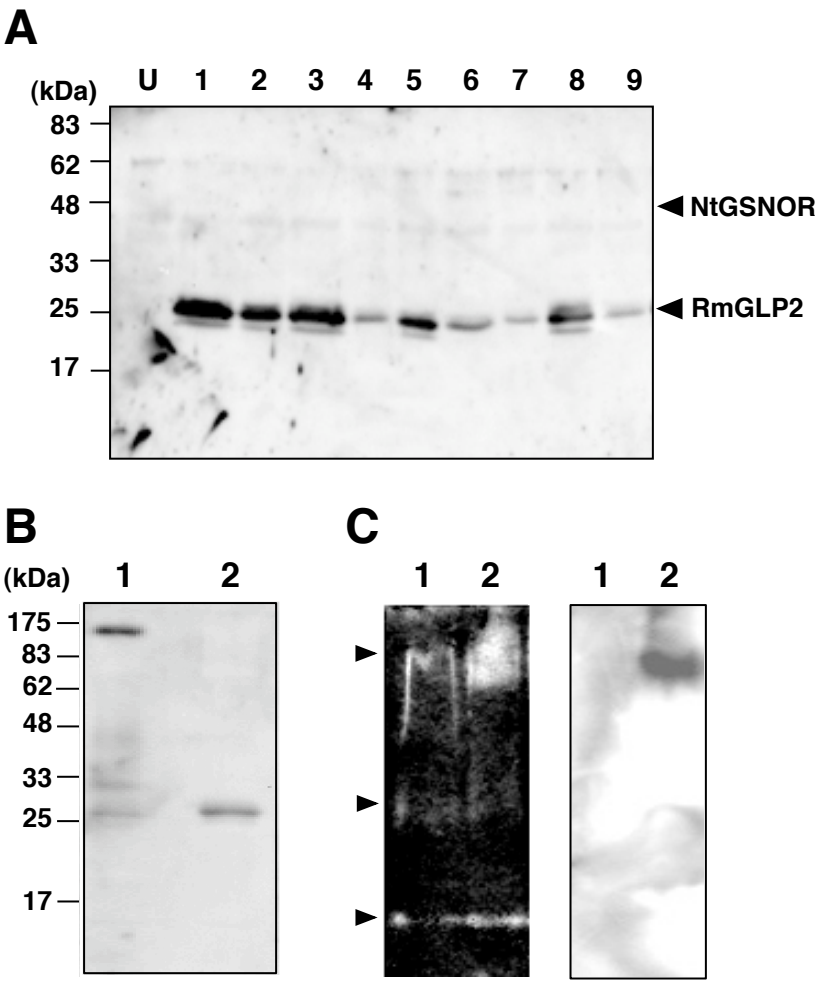


Supplementary Information

cDNA cloning of azalea ß-tubulin

A partial cDNA fragment for an azalea ß-tubulin, which served as an internal control in RT-qcPCR, was obtained by PCR amplification under the same conditions as described for GLP cloning, with the exception of an annealing temperature of 50°C. A set of degenerate primers,

5'-CA(G/A)CA(G/A)ATG(T/C)GGGA(T/C)(G/T)CIAA(G/A/C)AACATGATGTG-3' (sense) and

5'-CAT(G/A)TT(G/A)CT(T/C)TCIGC(T/C)TCIGT(G/A)AA(T/C)TCCAT(T/C)TC(G/A)TC C-3' (antisense), was designed on the basis of highly conserved regions among 15 cDNA and genomic sequences from various species (GenBank accession nos. within parentheses): *Anemia phyllitidis* (**X69185** and **X69186**), *Arabidopsis thaliana* (**M84700**, **M84701**, **M84703** and **M84704**), *Hordeum vulgare* (**Y09741**), *Zea mays* (**L10636**), *Pisum sativum* (**X54846**), *Oryza sativa* (**L19598** and **X78143**), *Glycine max* (**M21297** and **U12286**), and *Zinnia elegans* (**D63137** and **D63138**). A single amplified fragment with the expected size (375 bp) was sequenced to verify its identity (GenBank accession no. **AB272082**).

Bacterial production of recombinant proteins and antibody generation

The coding sequences for the putative mature form of RmGLP1 and RmGLP2, with truncation of the first 24 amino acid residues, which might constitute a putative signal peptide, were amplified by PCR with the sense (*RmGLP1*, 5'-TCATATGGATCCGGATATGCTCCAAGACGTTTGTGT-3'; *RmGLP2*, 5'-TCATATGGACCCGGATATGCTCCAAGATGTTTGTGC-3') and antisense (*RmGLP1*, CGGATCCAAGCAAACCAACAACATATCAACCCC; *RmGLP2*,

5’-CGGATCCGAAGAAAACCAAGAGCATGCCAATCCAAG-3’) primers that contained *Nde*I and *Bam*HI restriction sites (underlined), respectively. The PCR products were first cloned into pGEM-T Easy (Promega) for sequence verification and then mobilized as an *Nde*I-*Bam*HI fragment into the bacterial expression vector pET-16b (Novagen). The resulting plasmids were introduced into *Escherichia coli* BL21(DE3)pLysS cells and the recombinant proteins were induced as amino-terminal histidine-tagged forms by isopropyl-1-thio-ß-D-galactopyranoside. The recombinant RmGLP2 protein, obtained as a purified inclusion body from bacterial cells, was solubilized in SDS sample buffer [62.5 mM Tris-HCl (pH 6.8), 10% SDS, 5% ß-mercaptoethanol, 10% glycerol and 0.001% bromophenol blue] and subjected to SDS-PAGE using a 12% preparative polyacrylamide gel. The proteins were stained with Coomassie blue dye, after which the band corresponding to recombinant proteins was gel-purified and used directly as an antigen to raise rabbit polyclonal antibodies.

Construction of a plant expression vector and transformation of tobacco BY-2 cells

The entire *RmGLP2* coding sequence was obtained by PCR using 5'-AATCTAGAATGGTTGCTCCTCGAAAACTCTACGTGGTGGT-3' (sense) and 5'-TTGAGCT*CTAATGATGATGATGATGATG*TGCTAGCTTTGACTTGAT-3' (antisense), with *Xba*I and *Sac*I restriction sites (underlined), respectively. The latter primer included the sequence for hexahistidine and a stop codon (italicized) after the last amino acid codon, allowing a fusion with a histidine tag on the carboxyl-terminus of the encoded protein. Following sequence confirmation, the PCR product was introduced as an *Xba*I/*Sac*I fragment into the binary vector pIG121-Hm [12]. The resulting plasmid was transferred into *Agrobacterium tumefaciens* EHA101, which was used to transform tobacco BY-2 cells. Selection for transgenic BY-2 calli was performed for 2 weeks on solid Linsmaier and Skoog

medium supplemented with 50 mg l^{-1} hygromycin, 200 mg l^{-1} kanamycin, and 250 mg l^{-1} carbenicillin. Emerging transgenic calli were transferred to fresh solid medium containing hygromycin and kanamycin, and grown to appropriate density.

Genomic PCR

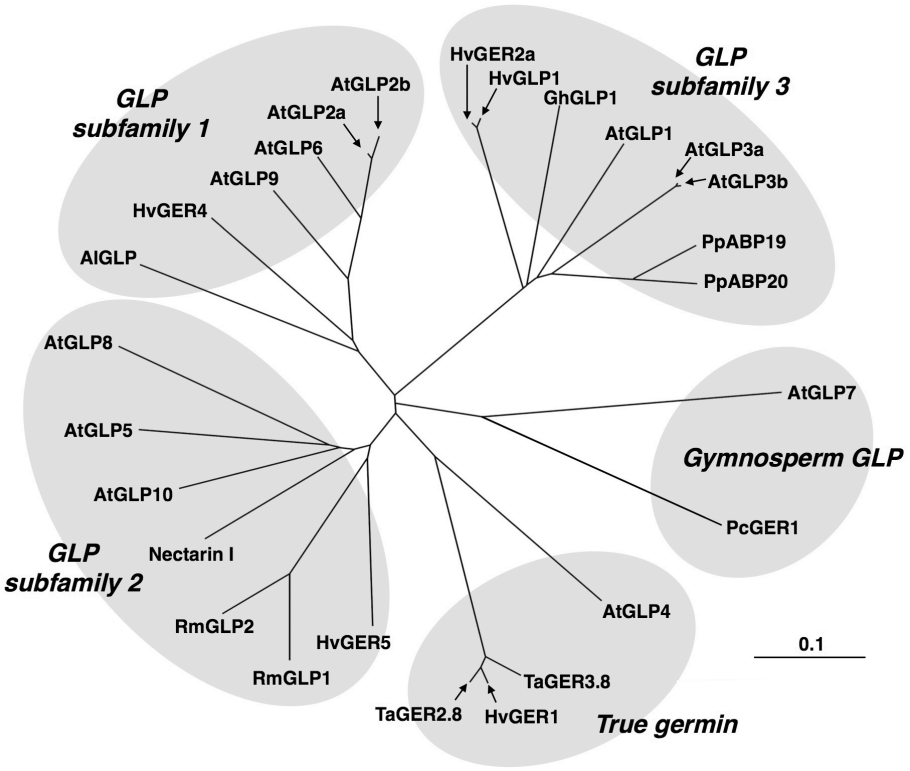
PCR was performed using DNA from antibiotic-resistant tobacco cells with the *RmGLP2*::His_6-specific primers as described above. The region spanning exons 3 and 4 of a tobacco nitrite reductase gene, *NtNii1* (GenBank accession no. **X66145**), was co-amplified as internal control in the same PCR reaction using the gene-specific primers, 5'-TGTGGGTGGGTTCTTCAGCG-3' (sense) and 5'-GGTGCAGTTCCACGAGAAAGAGAAG-3' (antisense). The PCR products were run on a 1% agarose gel and then visualized by ethidium bromide staining. The expected sizes of amplified products were 0.7 and 1.0 kb for *RmGLP2* and *NtNii1*, respectively.

Legends to Supplementary Figures

Figure S1. Unrooted dendrogram of higher-plant germins and GLPs. The tree was constructed based upon ClustalX (version 1.8.1) multiple alignment using a neighbor joining method with 1,000 bootstrap trials. Bar = 0.1 amino acid substitution/site. Selected sequences are those for twelve Arabidopsis GLPs and other germin/GLP members that have been examined or characterized for biochemical functions (i.e., OxO, SOD, ADP-glucose pyrophosphatase/phosphodiesterase and auxin-binding activities) [8]. The sources and GenBank accession numbers are as follows: *Atriplex lentiformis* (AlGLP, **AB024338**), *Arabidopsis thaliana* (AtGLP1, **U75206**; AtGLP2a; **U75192**; AtGLP2b, **X91957**; AtGLP3a, **U75188**; AtGLP3b, **U75195**; AtGLP4, **U75187**; AtGLP5, **U75198**; AtGLP6, **U75194**; AtGLP7; **AF170550**; AtGLP8, **U75207**; AtGLP9, **Z97336**; AtGLP10, **AL138642**), *Gossypium hirsutum* L. (GhGLP1, **AF116537**), *Hordeum vulgare* (HvGER1, **DQ647619**; HvGER2a, **DQ647620**; HvGER4, **DQ647623**; HvGER5, **DQ647624**; HvGLP1, **Y15962**), *Nicotiana langsdorffii* x *Nicotiana sanderae* (Nectarin I, **AF132671**), *Pinus caribaea* (PcGER1, **AF039201**), *Prunus persica* (PpABP19, **U79114**; PpABP20, **U81162**), *Rhododendron mucronatum* (RmGLP1, **AB272079**; RmGLP2, **AB272080**) and *Triticum aestivum* (TaGER2.8, **M63223**; TaGER3.8, **M63224**).

Figure S2. Reactivity of an anti-RmGLP2 antibody with bacterially expressed recombinant proteins. After SDS-PAGE (12% gel) of whole cell lysates (25 ng per lane) from *E. coli* cells harboring various plasmids, protein-gel blots were incubated with an anti-RmGLP2 antibody. Lane 1, pET16b (empty vector); lane2, RmGLP1-encoding plasmid; lane 3, RmGLP2-encoding plasmid.

Supplementary Fig. 1（Kondo et al.)



Supplementary Fig. 2（Kondo et al.)