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Vol. 10(22), pp. 2274-2285, 28 May, 2015
DOI: 10.5897/AJAR2014.9306
Article Number: 8C04D9A53314
ISSN 1991-637X
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African Journal of Agricultural Research

Full Length Research Paper

Cloning and gene expression analysis of ascorbic acid biosynthesis enzymes in *Moringa oleifera*

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Received 4 November, 2014; Accepted 19 May, 2015

Moringa (*Moringa oleifera*), which is a semi-tropical plant, is used as food and for the production of medicines and oil products, because of a large amount of various nutrients including ascorbic acid (AsA). Although Moringa leaf has a high AsA content, the molecular mechanisms of AsA accumulation in Moringa have received little attention. In this study, we isolated Moringa cDNAs for enzymes, belonging to the major AsA biosynthesis pathway (Smirnoff-Wheeler pathway) in higher plants. The predicted amino acid sequences showed 70% or more similarity to those of *Arabidopsis*. Quantitative RT-PCR indicated that Moringa GDP-L-galactose phosphorylase (*GGP*) is most highly expressed in Moringa during leaf development and light exposure. A significant high promoter activity of the Moringa *GGP* gene was detected by promoter assay in *Arabidopsis* protoplast.

Key words: *Moringa oleifera*, ascorbic acid, biosynthesis enzymes, gene expression.

INTRODUCTION

Ascorbic acid (AsA), vitamin C is reported as the sum of AsA and dehydroascorbic acid (DHA), is an essential human nutrient. Vitamin C, however, cannot be endogenously synthesized in the human body due to the absence of the last enzyme in the AsA biosynthesis pathway (Chatterjee, 1973), and must instead be obtained from fruit and vegetables in the diet. AsA has a variety of physiological roles (Smirnoff and Wheeler, 2000). For example, through its antioxidant properties, AsA scavenges reactive oxygen species (ROS) that are produced by abiotic stresses such as light (Asada, 2006), high and low temperature (Suzuki and Mittler, 2006), and drought (Helena and Carvalho, 2008). AsA also plays major roles in cell growth, photosynthesis, and control of anthesis (Barth et al., 2006; Mano et al., 2004). Scrutiny of AsA biosynthesis has led to several proposed synthesis pathways in plants, and one major pathway is the Smirnoff-Wheeler pathway, in which AsA is synthesized via D-mannose and L-galactose (Kanter et al., 2005; Radzio et al., 2003; Wheeler et al., 1998; Wolucka et al., 2001; Zhang et al., 2008). The Smirnoff-Wheeler pathway has been characterized, and involves eight AsA biosynthesis enzymes, namely, phosphomannose isomerase, phosphomannomutase,

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GDP-D-mannose pyrophosphorylase (*GMP*), GDP-D-mannose-3',5'-epimerase (*GME*), GDP-L-galactose phosphorylase (*GGP*), L-galactose-1-phosphate phosphatase (*GPP*), L-galactose dehydrogenase (*GDH*), and L-galactono-1,4-lactone dehydrogenase (*GalLDH*) (Wheeler et al., 1998). The *vtc1* mutant of *Arabidopsis*, which is deficient in *GMP* gene, has 25% of wild-type AsA, and the *vtc2 Arabidopsis* mutant, which is deficient in *GGP* gene, has 10 to 20% of wild-type AsA (Conklin et al., 1999; Dowdle et al., 2007). Although other AsA biosynthesis pathways, such as the galacturonate and *myo*-inositol pathways, have been found to function in plant AsA biosynthesis (Agius et al., 2003; Lorence et al., 2004), the Smirnoff-Wheeler pathway appears to be predominant in higher plants.

Moringa (*Moringa oleifera* Lam), which is native to northwest India, is an important multi-purpose tree that is used as food and for the production of medicines and oil products (Morton, 1991). The many potential uses of Moringa have led to the recent publication of a number of reports discussing the use of seed- and leaf-powders and extracts for, among others, water purification, nutrition, and medicine (Anselme et al., 1995; Anwar and Bhanger, 2003; Bhuptawat et al., 2007). Moringa can grow rapidly in tropical areas as well as in soils with relatively low nutrients and low humidity (Morton 1991), and has a high leaf AsA content (Sreelatha and Padma, 2009), suggesting that it could serve as a valuable dietary source of vitamin C for populations in less-developed countries. However, an increase in AsA content is required to optimally utilize the already high production capacity in Moringa. To date, the molecular mechanisms through which AsA accumulates in Moringa have not been determined, and elucidation of these molecular mechanisms is essential for the future improvement of AsA content in Moringa leaf. In this study, Moringa cDNAs for AsA biosynthesis enzymes were identified and used to evaluate gene expression. Quantitative RT-PCR analysis indicated that *MoGGP* was most highly expressed in Moringa among biosynthesis gene of the six investigated. The 5'-upstream region of the *MoGGP* gene was determined, allowing investigation of *cis*-element(s) enhanced promoter activity using *Arabidopsis* protoplasts.

MATERIALS AND METHODS

Plant materials

Moringa plants were grown at 25°C in 16 h of light (light intensity; 55.6 μmol/s/m^2) and 8 h of dark in a greenhouse. To compare the AsA contents and mRNA expression levels of AsA biosynthesis enzymes in the leaves of Moringa and *Arabidopsis*, the small (leaf length; <10 mm), medium (leaf length; 10 to 15 mm) and large leaves (leaf length; >15 mm) were prepared from Moringa plant. To test the effects of light on mRNA expression levels of AsA biosynthesis enzymes, leaf discs (8 mm diameter) were prepared from Moringa small leaves using a biopsy punch (8.0 mm, Kai industries). Leaf discs were floated on water in a petri dish, incubated in the dark overnight, and were then exposed to continuous light treatment (100 μmol/s/m^2), or were left to continuous darkness (0 μmol/s/m^2) at 25°C for 24 h. After light and dark treatment, discs were assayed for AsA content and mRNA expression levels of AsA biosynthesis gene.

Arabidopsis thaliana cv. Columbia seeds were placed on soil in a pot (8 cm wide by 7.5 cm high). The seedlings were soil-cultivated in a plant growth incubator at 25°C in 16 h of light (66.7 μmol/s/m^2) and 8 h of dark for 3 weeks. To measure AsA contents and mRNA expression levels AsA biosynthesis enzymes, rosette leaves were used.

Determination of ascorbic acid content

Total vitamin C content, as ascorbic acid content, was determined using an ascorbate oxidase method. Moringa and *Arabidopsis* leaves were harvested and ground in liquid nitrogen. The powdered tissues (100 mg) were homogenized in 1.0 ml of cold 6.0% (v/v) perchloric acid and centrifuged at 12,000 × g for 10 min at 4°C. To determine total vitamin C content, supernatants (350 μl) were combined with 110 μl of 1.25 M K_2CO_3 and the volume brought to 525 μl with distilled water. The extract (420 μl) was combined with 4 μl of 1 M dithiothreitol, incubated in the dark at 30°C for 30 min, and then centrifuged at 12,000 × g for 10 min. The supernatant (50 μl) was then combined with 446 μl of 200 mM succinate buffer and the absorbance was immediately measured at 265 nm. The absorbance at 265 nm was remeasured 30 s after the addition of 2.5 U of ascorbate oxidase. Total vitamin C content was calculated using a standard curve.

Reverse transcription-polymerase chain reaction

Total RNA was isolated from Moringa leaves using an RNeasy Plant Mini Kit with DNase I (Qiagen) according to the manufacturer's protocol. First-strand cDNA was synthesized from total RNA using a ReverTra Ace kit (Toyobo) and an oligo$(dT)_{20}$ primer. The cDNA was used as a template for polymerase chain reaction (PCR) using KOD Dash (Toyobo). Primers for AsA biosynthesis genes were designed based on the conserved amino acid sequences of plant AsA biosynthesis enzymes. The sequences for primers were used from the cDNA sequences of *Arabidopsis thaliana* AsA biosynthesis enzymes, and are listed in Supplementary Table 1. After initial denaturation for 2 min at 94°C, 40 cycles of amplification were carried out with 10 s denaturation at 98°C, 30 s annealing at 64°C for GalLDH, 56°C for GDH, GPP, GGP and GME or 55°C for GMP, and 60 s extension at 72°C. The primary PCR products were used as templates for nested PCR with additional gene-specific primers. Nested PCR was performed using KOD Dash, with the following thermocycler conditions: initial denaturation for 2 min at 94°C, followed by 40 cycles of 10 s denaturation at 98°C, 30 s annealing at 58°C, and 60 s extension at 72°C. PCR products were subcloned into the pGEM-T Easy vector (Promega, Madison, WI) and sequenced using an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems) with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's protocol.

5'- and 3'- rapid amplification of cDNA ends

Gene-specific primers (Supplementary Table 1) were designed and used for 5'- and 3'-rapid amplification of cDNA ends (RACE) to determine the nucleotide sequences of partial cDNA fragments. For 3'-end amplification, single-stranded cDNA was synthesized from total RNA (500 ng) using an oligo $(dT)_{17}$ adaptor primer and

ReverTra Ace. PCR for 3'-RACE was performed using KOD FX (Toyobo) with an adaptor primer and gene-specific primers.

After initial denaturation for 2 min at 94°C, 35 cycles of amplification were carried out with 10 s denaturation at 98°C, 30 s annealing at 55°C and 60 s extension at 68°C. The primary PCR product was used as templates for nested PCR, using an adaptor primer and a gene-specific primer and the same thermocycler conditions for the primary PCR. The resultant PCR products were subcloned and sequenced as described above. For 5'-RACE, circularized cDNA was synthesized from total RNA. Total RNA (5 μg) was reverse-transcribed using ReverTra Ace and a 5'-end phosphorylated oligo(dT)$_{17}$ primer. After hydrolysis of total RNA with RNase H (TaKaRa) at 30°C for 60 min, cDNA was circularized by ligation with T4 RNA Ligase (TaKaRa) at 15°C overnight. PCR was performed with KOD Dash and primers (GPP, oKT066 and oKT087; GGP, oKT064 and oKT065), using the circularized cDNA as templates. PCR conditions were as follows: 40 cycles of 30 s denaturation at 94°C, 10 s annealing at 55°C for GPP or 60°C for GGP, and 60 s at 72°C. PCR products were subcloned and sequenced as described above.

Cloning and sequencing of the Moringa GGP genomic sequence

The genome sequence of MoGGP was determined using gene-specific primers (Supplementary Table 1) designed against the MoGGP cDNA sequence. Genomic DNA was isolated from Moringa leaves using a DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. PCR was performed using KOD Dash with gene-specific primers, with 40 cycles of 30 s denaturation at 94°C, 10 s annealing at 60°C, and 60 s extension at 72°C. PCR products were subcloned and sequenced as described above. The 5'-upstream region of the MoGGP gene was isolated using the cassette-ligation mediated PCR method with an LA PCR *in vitro* Cloning Kit (TaKaRa). After digestion of genomic DNA with EcoRI or XbaI, the cleaved genomic DNA fragments were ligated to the appropriate double-stranded DNA cassette. Primary PCR was performed with a cassette-specific primer (C1) and a gene-specific primer, using KOD FX Neo reagents (Toyobo). PCR conditions were as follows: initial denaturation for 2 min at 94°C, 40 cycles of 10 s denaturation at 98°C, 30 s annealing at 64°C, 60 s extension at 68°C. Subsequent nested PCR used the primary PCR product as templates, alongside a cassette-specific primer (C2) and a gene-specific primer, and used the same thermocycler conditions as the primary PCR. PCR products were subcloned and sequenced as described above.

Real-time PCR

For internal reference of quantitative RT-PCR, partial cDNA fragment of Moringa rRNA was cloned by RT-PCR as described above. Primers (Supplementary Table 1) for Moringa rRNA were designed from the conserved sequences of plant rRNA; *Arabidopsis* (Acc. No. X52320), *Brassica napus* (Acc. No. D10840), *Cercidiphyllum japonicum* (Acc. No. AF274639) and *Disanthus cercidifolius* (Acc. No. AF274645). Reaction conditions were as follows: initial denaturation 30 s for 95°C, followed by 30 cycles of 5 s at 95°C and 10 s at 60°C. The resultant PCR products were subcloned and sequenced. The cDNA sequences are deposited in GeneBank under an accession number LC005430.

Total RNA was isolated from Moringa leaves using an RNeasy Plant Mini Kit with DNase I (Qiagen). Single-stranded cDNA was synthesized from RNA (500 ng) using the ReverTra Ace qPCR RT Kit (Toyobo), according to the manufacturer's protocol. Real-time PCR quantitation of transcripts of AsA biosynthesis enzymes was performed using Chrome4 (BioRad) and SsoFast EvaGreen Supermix (BioRad), according to the manufacturer's protocol. Reaction conditions were as follows: initial denaturation 30 s for 95°C, followed by 30 cycles of 5 s at 95°C and 10 s at 60°C with respective gene-specific primers (Supplementary Table 1). From the sequences of Moringa (Acc. No. LC005430) and *Arabidopsis* (Acc. No. X52320) rRNA, gene-specific primers were designed and used as respective internal reference. Transcript levels were determined using standard curves generated with DNA samples of known concentration. Normalization was performed by dividing Moringa AsA biosynthesis gene transcript levels with those of rRNA. PCR specificity was assured by examining the melting curve between 65°C and 95°C every 2 s with increments of 0.2°C, and by use of agarose gel electrophoresis to check for a single amplifying band. Real-time PCR experiments were performed in triplicate.

Promoter assay

The 5'-upstream region of the *MoGGP* gene was amplified by PCR using primers designed for In-Fusion PCR cloning, according to the manufacturer's instructions. For the *Arabidopsis GGP* promoter, 878 bp upstream of the *AtGGP* initiation codon was used. Amplified fragments were subcloned upstream of the *Renilla* luciferase coding sequence using In-Fusion PCR cloning. Preparation and transformation were performed using *Arabidopsis* protoplasts. Briefly, *Arabidopsis* protoplasts were prepared from rosette leaves of approximately 3-week-old *Arabidopsis thaliana* (ecotype Columbia) plants using cellulase "onozuka" R10 (Yakult Pharmaceutical Industry Co., Ltd.) and macerozyme R10 (Yakult Pharmaceutical Industry Co., Ltd.). Isolated protoplasts were suspended in MMg (0.4 M mannitol, 15 mM $MgCl_2$, and 4 mM MES pH 5.7) and used for protoplast transformation, which was performed in a 96-well plate (96-well U-bottom plate, Thermo SCIENTIFIC) using polyethylene glycol. Plasmid (1 pmol) expressing full-length *Renilla* luciferase under the control of the *MoGGP* promoter was used to transfect *Arabidopsis* protoplasts (3.0×10^5 cells). Plasmid (0.5 pmol) expressing full-length click beetle red luciferase under the control of the cauliflower mosaic virus 35S promoter was co-transformed for use as an internal reference. Luciferase luminescence was measured using a microplate luminometer (ARVOx4 2030 Multilabel Reader, Perkin Elmer, MA). The luminescence signals in each well were integrated for 3 s, 20 min after adding ViviRen (Promega) in each well. Luciferase luminescence was normalized for transformation efficiency by dividing the relative light units (RLU) of *Renilla* luciferase by the RLU of beetle red luciferase. Beetle luciferase activity was assessed by measuring luminescence through a red filter (effect filter 106, Always CO., LTD), with a 3 s integration period after the addition of the substrate.

RESULTS AND DISCUSSION

Isolation of cDNA clones encoding Moringa ascorbic acid biosynthesis enzymes

Reverse transcription (RT)-PCR was performed using primers designed from the cDNA sequences of *Arabidopsis* AsA biosynthesis enzymes. Partial cDNA fragments were isolated, and their nucleotide sequences were highly similar to those of the *Arabidopsis* AsA biosynthesis genes (data not shown). Rapid amplification of cDNA ends (RACE) methods were subsequently used to determine full-length cDNA sequences for Moringa

GGP and *GPP*. The cDNA of Moringa *GGP* (*MoGGP*) has a 1,320 bp open reading frame (ORF) that is predicted to encode a protein of 440 amino acids with a calculated molecular mass of 48,963 Da (Supplementary Figure 1). Primary structure analysis using the Conserved Domain Database (CDD) (Marchler-Bauer et al., 2013) suggested that *MoGGP* contains a histidine triad motif found in *Arabidopsis GGP* (*AtGGP*). The cDNA of Moringa *GPP* (*MoGPP*) has an 804 bp ORF that is predicted to encode a protein of 268 amino acids with a calculated molecular mass of 28,954 Da (Supplementary Figure 2). Primary structure analysis using CDD suggested that *MoGPP* has related domains of inositol monophosphatase family. *MoGGP* and *MoGPP* have 75 and 81% identities, respectively, to the *Arabidopsis* proteins, as indicated in Supplementary Table 2. We successfully identified the 3'-downstream sequences of Moringa *GMP*, *GME*, *GDH* and *GalLDH* cDNAs using 3'-RACE, but in this study we were unable to determine the 5'-upstream sequences of cDNAs using 5'-RACE. Comparison of the sequences between the *Arabidopsis* AsA biosynthesis enzymes and the partial predicted Moringa proteins indicated that high levels of similarity exist at the amino acid sequence level (Supplementary Table 2). We therefore concluded that the partial cDNAs isolated in this study are those encoding the Smirnoff-Wheeler pathway AsA biosynthesis enzymes in Moringa. The cDNA sequences are deposited in GeneBank under the following accession numbers: AB924374 for *MoGalLDH*, AB924375 for *MoGDH*, AB924376 for *MoGPP*, AB924377 for *MoGGP*, AB924378 for *MoGME*, and AB924379 for *MoGMP*.

Transcriptional levels of AsA biosynthesis enzymes in Moringa leaves

Genetic and biochemical studies indicate that the Smirnoff-Wheeler pathway is both ubiquitously expressed and is the dominant AsA biosynthesis pathway in higher plants (Conklin, 2001; Conklin et al., 2000; Wheeler et al., 1998; Wolucka and Van Montagu, 2007). Figure 1A shows that Moringa small (< 10 mm), medium (10 to 15 mm) and large (> 15 mm) leaves contained 10.9, 5.2 and 4.1 μmol/gFW (gram fresh weight) AsA, respectively. AsA content in *Arabidopsis* rosette leaf was 3.6 μmol/gFW. Sreelatha and Padma reported that Moringa leaf contained about 30.5 μmol/gFW AsA (Sreelatha and Padma, 2009). The Moringa plants used in this study were grown under fluorescent at 25°C in a green house. The comparative-low AsA contents in Moringa leaves used in this study, may be due to growth condition, such as light intensity, humidity or temperature, because AsA content in plant is subject to ambient growth condition (Davey et al., 2000). To evaluate AsA biosynthesis at transcriptional levels in Moringa leaves, mRNA levels of AsA biosynthesis enzymes isolated in this study were measured in small, medium and large leaves using quantitative RT-PCR (Figure 1B). The mRNAs encoding all six Moringa AsA biosynthesis enzymes were expressed during Moringa leaf development, indicating that the Smirnoff-Wheeler pathway is likely functional during Moringa leaf development. As indicated in Figure 1B and 1C, the transcriptional patterns of the Moringa and *Arabidopsis* genes tested in this study were different from each other. Of the biosynthesis genes tested, the *MoGGP* mRNA was expressed at the highest levels at all developmental stages. The *Arabidopsis vtc2* mutant is defective in the production of *GGP*, and contains only 10 to 25% of the AsA of a wild-type plant (Conklin et al., 1999; Dowdle et al., 2007). On the other hand, estrogen inducible transiently overexpression of *AtGGP* resulted in an increase in AsA contents (Yoshimura et al., 2014). This indicates that *AtGGP* is a key enzyme in *Arabidopsis* AsA biosynthesis. In acerola, which contains extremely abundant AsA, expression levels of *GGP* mRNA were the highest of the AsA biosynthesis mRNAs tested (Badejo et al., 2009). The acerola data, alongside our findings here, suggest that *MoGGP* may be the predominant enzymes contributing to AsA accumulation in Moringa leaves.

Effects of light exposure on mRNA levels of AsA biosynthesis enzymes in Moringa leaf

Exposure of plants to light causes an increase in AsA levels (Cruz-Rus et al., 2010; Fukunaga et al., 2010; Li et al., 2009; Yabuta et al., 2007). Multiple AsA biosynthesis enzymes are thought to participate in light-triggered AsA biosynthesis in plants. We evaluated the effects of light on AsA production in Moringa by measuring mRNA levels in leaf discs treated with continuous light exposure. As indicated in Figure 2A, AsA content was significantly higher in leaf discs illuminated at 100 μmol/s/m^2 light than in those incubated in the dark (0 μmol/s/m^2). Quantitative RT-PCR analysis revealed that *MoGGP* was the only AsA biosynthesis gene to display significantly increased expression on exposure to light treatment (Figure 2B). In *Arabidopsis* transiently overexpressing *AtGGP*, the increase in AsA levels was enhanced under continuous light (Yoshimura et al., 2014). The finding that only the *MoGGP* mRNA levels increase with light exposure suggests that this is the dominant enzyme involved in light-triggered AsA biosynthesis in Moringa.

Sequence and activity of the Moringa GGP promoter

The *MoGGP* gene sequence (Acc. No. AB924665) was determined through amplification of genomic DNA using primers designed from the *MoGGP* cDNA. The *MoGGP* gene structure was determined by comparing the genomic and cDNA sequences, and was found to comprise seven exons (Figure 3A).

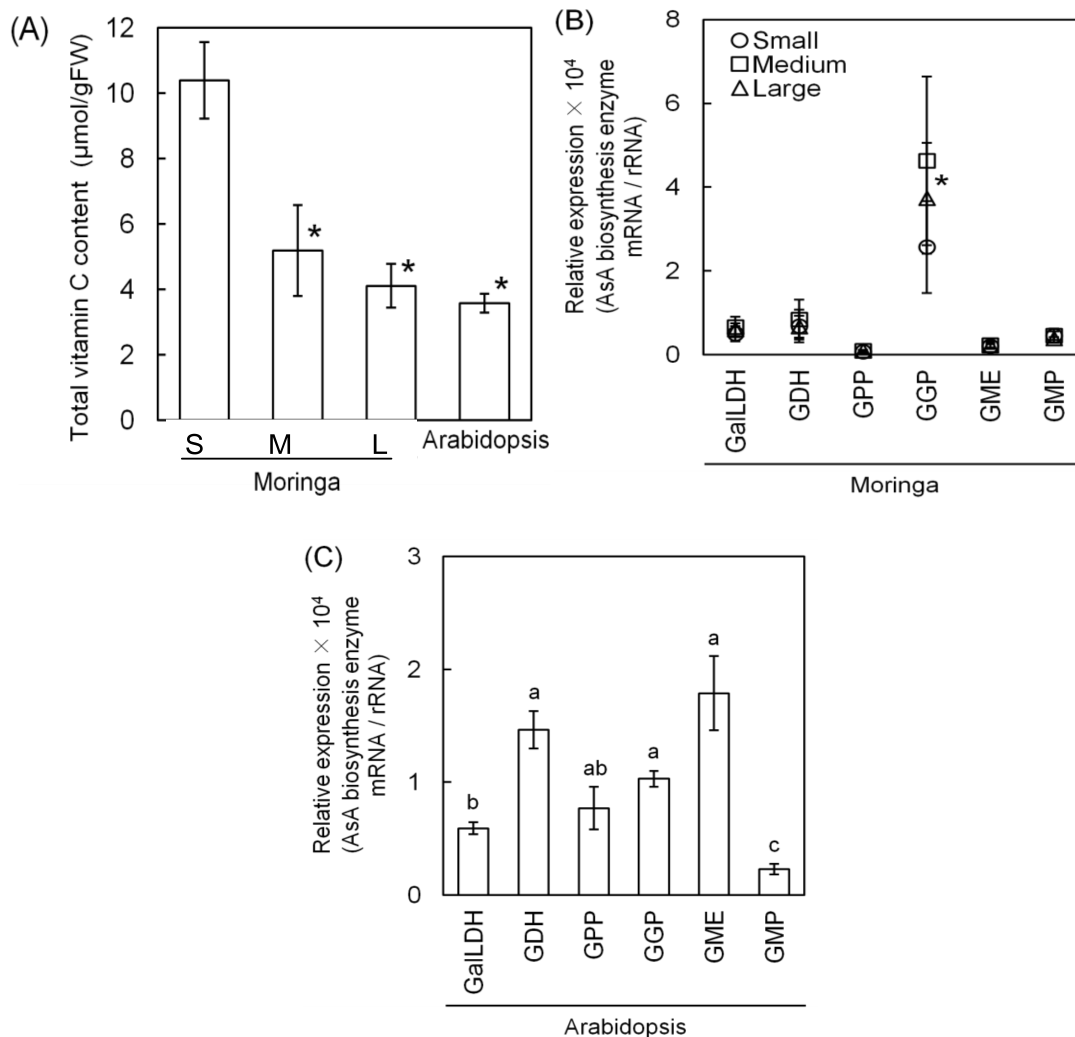

Figure 1. The AsA contents and mRNA expression levels of AsA biosynthesis enzymes in Moringa leaves. (A) The extracts were prepared from the small (S, < 10 mm), medium (M, 10 to 15 mm) and large (L, > 15 mm) Moringa leaves, and the rosette leaves of *Arabidopsis*. The total vitamin C contents, as the AsA contents, were determined in the extracts using ascorbate oxidase as described in materials and methods section. Bars represent means ± SE (n = 3). Asterisks indicate significant difference as compared to total vitamin C content in small Moringa leaves ($p < 0.05$). (B) Total RNA (500 ng) was extracted from the Moringa leaves. The mRNA levels of AsA biosynthesis enzymes in the Moringa leaves were measured using total RNA by quantitative RT-PCR as described in materials and methods section. The mRNA expression levels were normalized with rRNA levels. Bars represent means ± SE (n = 3). (C) The mRNA levels of AsA biosynthesis enzymes in the *Arabidopsis* leaves were measured by quantitative RT-PCR. The mRNA expression levels were normalized with rRNA levels. Bars represent means ± SE (n = 3). Values followed by the same letter are not significantly different ($p <0.05$).

The genomic and cDNA sequences were compared between *MoGGP* and *AtGGP*, which indicated that both exon number and length were well conserved. This suggests that the *GGP* gene likely arose prior to the divergence of *Arabidopsis* and Moringa. We also sequenced ~0.8 kb of the 5'-region upstream of the *MoGGP* initiation codon (Figure 3B). The 215 bp directly upstream of the initiation codon was identical to the 5'-UTR of the *MoGGP* cDNA, indicating that the transcription start site (TSS) of the *MoGGP* gene is more than 200 bp upstream of the initial ATG. The 5'-upstream region was searched for transcription factor recognition sites using the PLACE program (Higo et al., 1999). Several consensus elements were found, including two SORLIP1AT (GCCAC; Hudson and Quail, 2003) and IBOX (GATAAG; Giuliano et al., 1988) motifs within a 600 bp promoter region. Expression of *MoGGP* was induced by light exposure, suggesting that these sequences are likely to function as light responsible *cis*-elements. We could not, however, find TATA or CAAT boxes upstream

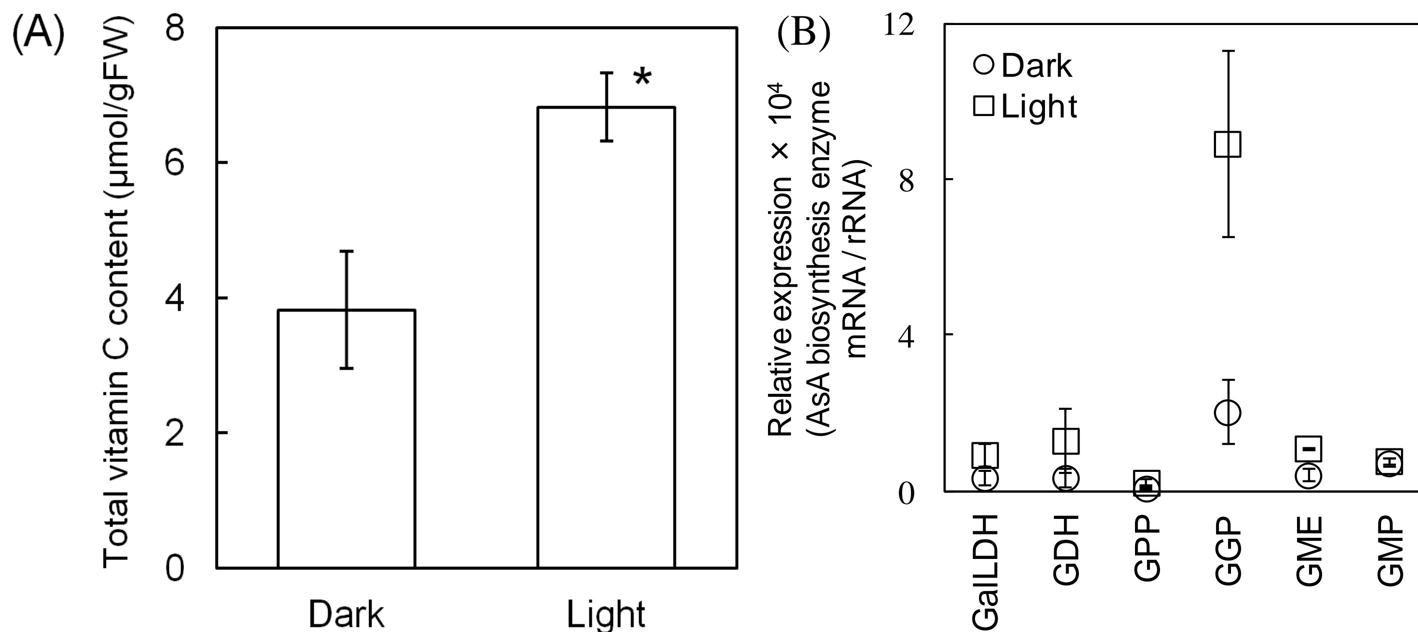

Figure 2. The AsA contents and mRNA expression levels of AsA biosynthesis enzymes in response to light exposure. (A) Before light treatment, leaf discs were floated on water in the dark overnight. The total vitamin C contents of Moringa leaf discs were measured at 24 h after continuous light (100 µmol/s/m^2) or darkness (0 µmol/s/m^2). An asterisk indicates significant difference as compared to the total vitamin C content in the leaf discs treated with darkness. (B) The mRNA levels of AsA biosynthesis enzymes in the Moringa leaf discs treated with light were measured by quantitative RT-PCR. The mRNA expression levels were normalized with rRNA levels. Bars represent means ± SE (n = 3). Asterisks indicate significant difference as compared to the mRNA expression level in the leaf discs treated with darkness (p <0.05).

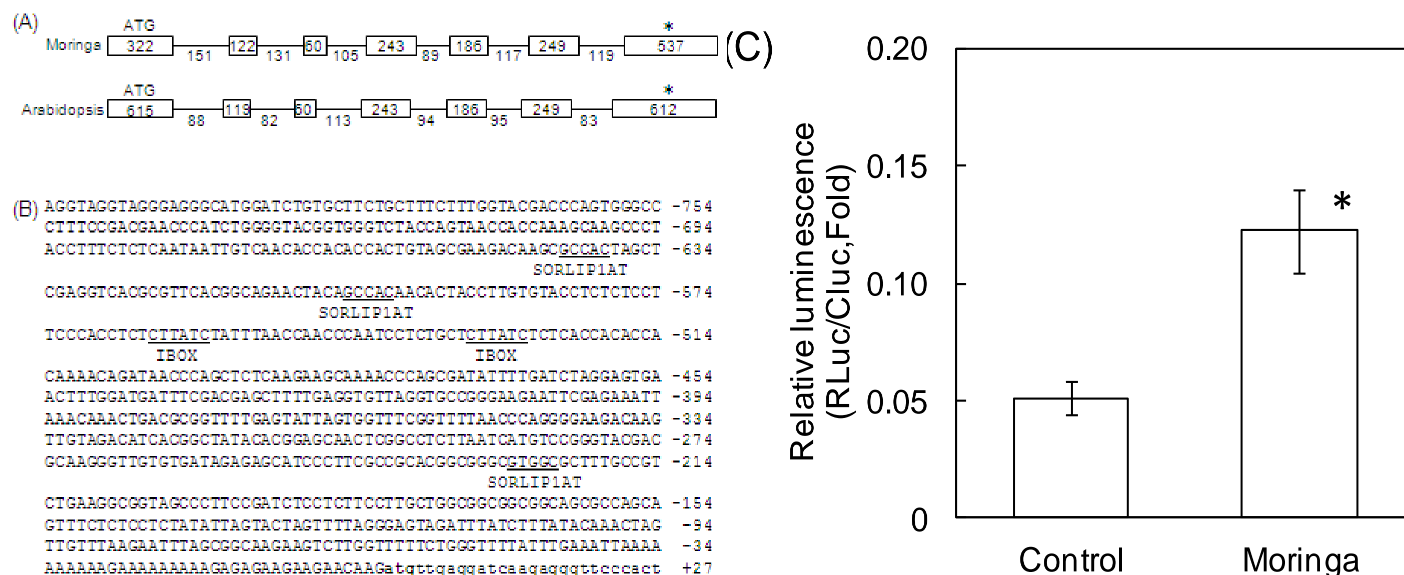

Figure 3. The sequence and promoter activity of Moringa GDP-L-galactose phosphorylase. (A) The length of each exon and intron. The exons and the introns are showed by white boxes and black lines respectively. The respective lengths are indicated in bp. The diagram is not to scale. ATG and asterisks indicate the initiation codon and the stop codon respectively. (B) The nucleotide sequence of the 5'-upstream region of *MoGGP* gene. The position of the initiation codon (ATG) is represented by +1 at the adenine nucleotide. Underlining indicates sequences that are similar to *cis*-element reported previously. (C) Luciferase activities driven by the Moringa *GGP* promoters. The *MoGGP* promoter was subcloned into the upstream of the *Renilla* luciferase. In promoter assays, after transfection with 1 pmol of the plasmid and 0.5 pmol of a plasmid containing the click beetle red luciferase gene driven by the cauliflower mosaic virus 35S promoter to *Arabidopsis* protoplasts, luciferase activities in the protoplasts were measured using a microplate luminometer described in the materials and methods section. For each transfection, *Renilla* luciferase activity was normalized with click beetle red luciferase activity. Bars represent means ± SE (n = 3). Asterisks indicate significant difference as compared to control (p <0.05).

of the *MoGGP* gene, and the core promoter was a minimum promoter region capable of initiation basal transcription. By contrast, the PLACE program located two TATA boxes at 292 and 344 bp upstream of the *Arabidopsis GGP* TSS (data not shown), suggesting that different transcription factors are involved in *GPP* promoter activation in the two species. We tested the *cis*-element promoter activity of *MoGGP* with a transient expression assay using *Arabidopsis* protoplasts. Protoplasts were isolated from *Arabidopsis* leaves and transiently transfected with *MoGGP*-LUC reporter construct. Protoplasts transfected with *MoGGP*-LUC exhibited 2-fold higher luminescence intensities than mock-treated protoplasts (Figure 3C). Although TATA boxes do not appear to be present in the *MoGGP* promoter region determined in this study, an unknown *MoGGP* promoter element may be driving expression. Further investigation of the promoter regions upstream of *MoGGP* is necessary to address this question.

In this study, we wished to examine the biosynthesis pathways underlying the high levels of AsA found in Moringa leaves. We identified six novel Moringa genes putatively encoding AsA biosynthesis enzymes in the Smirnoff-Wheeler pathway, and suggest that these genes play a major role in AsA biosynthesis during leaf development and under light conditions. Unlike acerola, in which several enzymes are important for AsA synthesis, *MoGGP* seems to be the major important enzyme regulating AsA content in Moringa. No TATA boxes were found in the 5'-region upstream of *MoGGP*. We found that the putative *cis*-elements may be involved in the light response, but we were unable to provide direct evidence of *cis*-element(s) enhanced gene expression in this study. Further investigation of more distal 5'-regions is necessary to determine the elements through which *MoGGP* expression is regulated, providing valuable information concerning the mechanisms underlying *GGP* transcription in Moringa.

Abbreviations: AsA, Ascorbic acid; ***GalLDH*,** L-galactono-1,4-lactone dehydrogenase; ***GDH,*** L-galactose dehydrogenase; ***GGP*,** GDP-L-galactose phosphorylase; ***GME*,** GDP-D-mannose-3',5'-epimerase; ***GMP*,** GDP-D-mannose pyrophosphorylase; ***GPP*,** L-galactose-1-phosphate phosphatase; **RACE,** rapid amplification of cDNA ends; **RLU,** relative light units; **ROS,** reactive oxygen species; **TSS,** transcription start site.

Conflict of Interest

The author(s) have not declared any conflict of interest.

ACKNOWLEDGEMENTS

This research was supported by the MEXT project “Practical Research on Sustainable Development in Central American and Caribbean Countries”. The sequence analysis was carried out at the Analysis Center of Life Science, Natural Science Center for Basic Research and Development, Hiroshima University.

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SUPPLEMENTARY

Supplementary Figure

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cggtagcccttccgatctcctcttccttgctggcggcggcggcagcgccagcagtttctctcctctatat 70
tagtactagttttagggagtagatttatctttatacaaactagttgtttaagaatttagcggcaagaagt 140
cttggtttttctgggttttatttgaaattaaaaaaaaaagaaaaaaaagagagaagaagaacaagATGTT 210
                                                                  M  L

GAGGATCAAGAGGGTTCCCACTGTTGTCTCGAATTACCAGAAGGAAGAGGGGGAAGAAGGTGCTCGCCGA 280
  R  I  K  R  V  P  T  V  V  S  N  Y  Q  K  E  E  G  E  E  G  A  R  R

GAAGGCGGCTGTGGCCGGAATTGCCTCAACAAGTGTTGCATACAAGGGGCGAAGATTCCATTATATGTTT 350
 E  G  G  C  G  R  N  C  L  N  K  C  C  I  Q  G  A  K  I  P  L  Y  V

TCAAAGGGCTGAACAAGACTGGTGGCAGCAAGGGTGTGCTTGGGCATGAGAACGGGGAGCCTCCTGTTGC 420
F  K  G  L  N  K  T  G  G  S  K  G  V  L  G  H  E  N  G  E  P  P  V  A

CTTTCTTGAGTCACTCCTTCTTGGGGAGTGGGAGGATCGCTCGGAAAGAGGGCTTTTTCGGTATGATGTC 490
  F  L  E  S  L  L  L  G  E  W  E  D  R  S  E  R  G  L  F  R  Y  D  V

ACTGCCTGTGAAACCAAGGTGATCCCGGGTGACTATGGCTTCATAGCCCAGCTGAACGAGGGCCGCCATC 560
 T  A  C  E  T  K  V  I  P  G  D  Y  G  F  I  A  Q  L  N  E  G  R  H

TCAAGAAGAGGCCAACTGAATTCCGTGTTGATAAGGTCCTGCAGCCTTTTGATGGGAACAAATTCAACTT 630
L  K  K  R  P  T  E  F  R  V  D  K  V  L  Q  P  F  D  G  N  K  F  N  F

CACCAAAGTTGGGCAGGAAGAGGTACTCTTCCAGTTTGAAGCAAGTGAAGATGGTGAAGTTCAGTTCTTT 700
  T  K  V  G  Q  E  E  V  L  F  Q  F  E  A  S  E  D  G  E  V  Q  F  F

CCAAGTGCACCCATTGATGTTGAGAATTCTCCTAGTGTTGTTGCTATAAATGTTAGTCCTATTGAGTATG 770
 P  S  A  P  I  D  V  E  N  S  P  S  V  V  A  I  N  V  S  P  I  E  Y

GGCATGTGCTGTTGATCCCTCGTGTTCTTGAGTGCTTGCCACAGAGGATTGACCGTGACAGCTTCTTGCT 840
G  H  V  L  L  I  P  R  V  L  E  C  L  P  Q  R  I  D  R  D  S  F  L  L

TGCCCTTCACATGGCAGCTGAAGCTGGAAATCCATATTTCCGGTTGGGTTACAACAGTTTAGGTGCATTT 910
  A  L  H  M  A  A  E  A  G  N  P  Y  F  R  L  G  Y  N  S  L  G  A  F

GCTACTATCAATCATCTGCACTTCCAGGCTTATTACCTGGCTATGCCCTTTCCAGTTGAGAAGGCTCCCA 980
 A  T  I  N  H  L  H  F  Q  A  Y  Y  L  A  M  P  F  P  V  E  K  A  P

CCAAGAAGATTACCACCACTGATGGTGGGGTGAGGATCTCAGAACTTTTGAATTACCCAGTTAGAAGTCT 1050
T  K  K  I  T  T  T  D  G  G  V  R  I  S  E  L  L  N  Y  P  V  R  S  L

TGTCTTCGAGGACGGTGAAACTGTGCAAGACTTATCCAACACGGTATCGGATGCCTGCATTTGCCTCCAA 1120
  V  F  E  D  G  E  T  V  Q  D  L  S  N  T  V  S  D  A  C  I  C  L  Q

AACAGCAACATTCCTTACAATGTCCTCATTGCTGATTGTGGAAACCGGGTCTTTGTCTTCCCGCAGTGCT 1190
 N  S  N  I  P  Y  N  V  L  I  A  D  C  G  N  R  V  F  V  F  P  Q  C

ATGCTGAGAAACAAGCTCTTGGGGAAGTGAGTCCTGAGCTTCTGGATACCCAAGTTAACCCTGCTGTTTG 1260
Y  A  E  K  Q  A  L  G  E  V  S  P  E  L  L  D  T  Q  V  N  P  A  V  W

GGAAATCAGTGGGCATATGGTATTGAAGAGGAAGAAGGATTATGAAGAGGCATCCGAGGAAAATGCTTGG 1330
  E  I  S  G  H  M  V  L  K  R  K  K  D  Y  E  E  A  S  E  E  N  A  W

AGGCTCCTTGCTGAGGTGTCTCTTTCTGAAGAGAGGTTCAGGGAAGTGAGTGCTCTGATCTTTGAGGCCA 1400
 R  L  L  A  E  V  S  L  S  E  E  R  F  R  E  V  S  A  L  I  F  E  A

TTGCTTGCAGTGAAAATGGTGAGGCAAGCAATGAACAGAGCTCGGTGAACAAAAATGTGCATGCCATAAA 1470
I  A  C  S  E  N  G  E  A  S  N  E  Q  S  S  V  N  K  N  V  H  A  I  K

GAAGAGCTCTCACTCTGCTATAGTGACTGGGACACAAGAATGCCTGGTTCTCCAGTAGaatcgggtgttt 1540
  K  S  S  H  S  A  I  V  T  G  T  Q  E  C  L  V  L  Q  *

ggttatcggacttgctgtgtttgtgattctgaagtatgagactttgcacatgagatcatatgggtttgtg 1610
gcactagtggcctaaataagcaaacctggtttgcactggttgtggtagtcctttgtgtttatgtgttgtg 1680
cttgttagcctggtgatgtactgtgtggagccttgttcaagtactttgttgtataatgtttgctgctata 1750
agctaatgaatttgtcttattctgtaaaaaaaaaaaaaaaa                              1791
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Figure 1. Nucleotide and deduced amino acid sequences of *MoGGP*. An asterisk shows the termination codon.

```
cgatatttttcatgtgatacgtgaaaacctccaacacagagcacaacctatcacccatcagcttcctcc 70
ccccctggcgtatcactgatcaacccagtgaagaaaaaaATGGCTGAAAATTTTTCGCTGGAAGAATTCT 140
                                         M  A  E  N  F  S  L  E  E  F

TGGCATCTGCAGTTGATGCAGCTAAGAAAGCTGGCGAGATAATCCGTAAAGGATTTTACCAAACCAAACA 210
L  A  S  A  V  D  A  A  K  K  A  G  E  I  I  R  K  G  F  Y  Q  T  K  H

TGTGGAACATAAAGGCCAGGTAGATTTGGTCACAGAGACTGATAAAGCATGTGAGGATCTTGTATTTAAT 280
  V  E  H  K  G  Q  V  D  L  V  T  E  T  D  K  A  C  E  D  L  V  F  N

CATCTCAAGCAGCATTACCCCAATCATAAGTTCATTGGGGAAGAAACTACTGCTGCTTATGGTGCTACAG 350
 H  L  K  Q  H  Y  P  N  H  K  F  I  G  E  E  T  T  A  A  Y  G  A  T

AGCTGACTGATGAACCCACTTGGATAGTTGATCCTCTTGATGGAACGACTAACTTTGTCCATGGATTCCC 420
E  L  T  D  E  P  T  W  I  V  D  P  L  D  G  T  T  N  F  V  H  G  F  P

CTTTGTGTGCGTCTCTATTGGTCTCACGATCAAGAAGGTTCCTACAGTTGGTGTTGTGTACAATCCGATT 490
  F  V  C  V  S  I  G  L  T  I  K  K  V  P  T  V  G  V  V  Y  N  P  I

ATGGAAATTCGATTTACTGGCATCCACGGGAAAGGTGCTTTCTTGAATGGAAACCCTATAAAAGTATCAT 560
 M  E  I  R  F  T  G  I  H  G  K  G  A  F  L  N  G  N  P  I  K  V  S

CTAAAACTGAACTTGTCAAATCCCTTCTTGCAACTGAGGCTGGAACAAAACGGGATAAGGCAACTCTAGA 630
S  K  T  E  L  V  K  S  L  L  A  T  E  A  G  T  K  R  D  K  A  T  L  D

TGATTCGACGAACAGAATCAATAATCTGCTTTCCAAGGTGAGATCTCTTCGAATGAGTGGCTCGTGTGCA 700
  D  S  T  N  R  I  N  N  L  L  S  K  V  R  S  L  R  M  S  G  S  C  A

TTGAACCTCTGCGGAATTGCCTGTGGAAGGCTTGATCTGTTTTACGAACTTGGATTTGGTGGCCCATGGG 770
 L  N  L  C  G  I  A  C  G  R  L  D  L  F  Y  E  L  G  F  G  G  P  W

ATGTTGCTGGAGGTGCTGTTATTGTTAGAGAAGCTGGAGGACTTGTGTATGATCCATCTGGTAAAGATTT 840
D  V  A  G  G  A  V  I  V  R  E  A  G  G  L  V  Y  D  P  S  G  K  D  F

TGATATCACATCTCAGCGAGTTGCAGCTTCAAACCCTTCCCTGAAGGATTTGTTCGTGGAGGCTTTGCAG 910
  D  I  T  S  Q  R  V  A  A  S  N  P  S  L  K  D  L  F  V  E  A  L  Q

CTTTAAgaacgacagtgcacctaaagcctatgaagactagatgtagccgttatttaaatataatttaatc 980
 L  *

aagttctctcatttttccagcaatttctattcttaccatgaggattcaaaatgtatcatctctgagagag 1050
ttgatgacaacaattcttccaattggcatgtggccttattcctgtgccagattgctagtagaacctaatg 1120
gttaatatgtatttgtatcactttcaaattatcgcattgttgagctctaaaaaaaaaaaaaaaaaa     1186
```

Figure 2. Nucleotide and deduced amino acid sequences of *MoGPP*. An asterisk shows the termination codon.

Table 1. Primers used for cDNA cloning, quantitative RT-PCR and promoter assay.

Primer name	Oligonucleotide sequence (5'-3')	Purpose
oYF089	CATCTAGCTCGATGTGGCCT	GalLDH RT-PCR forward
oYF090	GCCCAATGTTCATACGCAGA	GalLDH RT-PCR reverse
oYF067	GTTGGTTTTGGTGCCTCTCCG	GDH RT-PCR 1st forward
oYF070	TCTTTACAGGCTCGAGAATAGCTTCAAC	GDH RT-PCR 1st reverse
oYF071	CACAAAGGCCAGGTGGATTTGG	GPP RT-PCR 1st forward
oYF074	GTCCCATGGACCACCGAAACC	GPP RT-PCR 1st reverse
oYF075	GGCTGTGGACGGAATTGCCTC	GGP RT-PCR 1st forward
oYF078	CCTCTTCAGTACCATGTGACCACT	GGP RT-PCR 1st reverse
oYF079	GCTGCTGATATGGGTGGTATGGG	GME RT-PCR, 3'-RACE 1st forward
oYF082	AGGAGCCCAACCAAGCTTTTC	GME RT-PCR, 3'-RACE 1st reverse
oYF083	GGAGGTTTTGGCACTCGCTTG	GMP RT-PCR 1st forward
oYF086	GTTATGCAACACAACCCCTCCATT	GMP RT-PCR 1st reverse
oYF068	CTCGGTATCAACTTCTTCGACACCT	GDH RT-PCR 2nd forward

Table 1. Contd.

oYF069	AGCAGGGTGCCATTCAGGAGG	GDH RT-PCR 2nd reverse
oYF072	TTCATAGGAGAAGAAACTACAGCTGCA	GPP RT-PCR 2nd forward
oYF073	TCCATTCAAGAATGCTCCTTTCCC	GPP RT-PCR 2nd reverse
oYF076	GCTCAGCTTAACGAGGGTCGT	GGP RT-PCR 2nd forward
oYF077	ACCTAGAGCCTGTTTCTCTGCGTA	GGP RT-PCR 2nd reverse
oYF080	TCGAGTGCTTGTATCTATCCAGAG	GME RT-PCR 2nd forward
oYF081	CAACCTGAGTACACCTTCAACACA	GME RT-PCR 2nd reverse
oYF084	GAAGTCGTCTTGGCCATTAACTACGAG	GMP RT-PCR 2nd forward
oYF085	CCGACCGTCGAGTGCCA	GMP RT-PCR 2nd reverse
oKT003	TCAAGCCGAAGCTGAGTTTT	GalLDH 3'-RACE 1st forward
oKT004	GGGATACAGAGTGGGATGGA	GalLDH 3'-RACE 2nd forward
oKT005	GGGTGTTGGTGTAATCAGTGC	GDH 3'-RACE 1st forward
oKT006	CACTTGCAATGGGACTCCTT	GDH 3'-RACE 2nd forward
oKT007	GGTTCCTACAGTTGGTGTTGTG	GPP 3'-RACE 1st forward
oKT008	TACTGGCATCCACGGGAAA	GPP 3'-RACE 2nd forward
oKT009	CCTCATTGCTGATTGTGGAA	GGP 3'-RACE 1st forward
oKT010	GTCTTTGTCTTCCCGCAGTG	GGP 3'-RACE 2nd forward
oKT027	TCAAAATATGGGGTGGTGGT	GMP 3'-RACE 1st forward
oKT028	CCACAGGGAAAGTTGAAAGG	GMP 3'-RACE 2nd forward
oKT066	ATGTGGCCTTATTCCTGTGC	GPP 5'-RACE forward
oKT087	TGCCTTATCCCGTTTTGTTC	GPP 5'-RACE reverse
oKT064	ACTGTGTGGAGCCTTGTTCA	GGP 5'-RACE forward
oKT065	TCAGTTGGCCTCTTCTGGAG	GGP 5'-RACE reverse
oKT200	TAGCCCTTCCGATCTCCTCT	Moringa GGP genomic DNA forward
oKT201	TACTTGAACAAGGCTCCACACA	Moringa GGP genomic DNA reverse
oKT118	AAAAGCTGTTCCCCCTGTCT	Moringa Real-time PCR GalLDH forward
oKT046	ACAAAGACGTCCACCTCCAC	Moringa Real-time PCR GalLDH reverse
oKT106	AATGTTGCTGCTGCCTCAGA	Moringa Real-time PCR GDH forward
oKT107	GATGTCAGCCCTGCTGGATT	Moringa Real-time PCR GDH reverse
oKT176	TTAGAGAAGCTGGAGGACTTGTG	Moringa Real-time PCR GPP forward
oKT177	CTTCATAGGCTTTAGGTGCACTG	Moringa Real-time PCR GPP reverse
oKT156	GGACACAAGAATGCCTGGTT	Moringa Real-time PCR GGP forward
oKT157	AGGCCACTAGTGCCACAAAC	Moringa Real-time PCR GGP reverse
oKT212	AGTGAGGAGCATAAGAGCTACCC	Moringa RealTime-PCR GME forward
oKT213	GGACAGGTATCAGGCCAATTAGT	Moringa RealTime-PCR GME reverse
oKT158	CGCATGTATATCCAGCAGCA	Moringa Real-time PCR GMP forward
oKT125	TCTCTTTGTGGGGCAGAACT	Moringa Real-time PCR GMP reverse
oKT240	GTAAACGGCGGGAGTAACTATG	Moringa, Arabidopsis RT-PCR Real-time PCR rRNA forward
oKT241	AGTCGGACTAGAGTCAAGCTCAA	Moringa, Arabidopsis RT-PCR Real-time PCR rRNA reverse
oKT159	CAACAACATGGTGGAAAAGC	Arabidopsis Real-time PCR GalLDH forward
oKT127	TGGATTCACTCATTCCTCACC	Arabidopsis Real-time PCR GalLDH reverse
oKT128	CAAGGAGATTTCGTCGGTGT	Arabidopsis Real-time PCR GDH forward
oKT129	TTCTTGATCCATCCCCAGAC	Arabidopsis Real-time PCR GDH reverse
oKT130	CCATCCGGTAAAGATTTGGA	Arabidopsis Real-time PCR GPP forward
oKT131	GACTTCATGCCCCTGTAAGC	Arabidopsis Real-time PCR GPP reverse
oKT132	CGAGTGCCTTGTCCTTCAGT	Arabidopsis Real-time PCR GGP forward
oKT133	ATGCCCACAACCATCATACA	Arabidopsis Real-time PCR GGP reverse
oKT134	AGCTTGGTTGGGCTCCTAAT	Arabidopsis Real-time PCR GME forward
oKT135	ACCACCTTTGATGACCCGTA	Arabidopsis Real-time PCR GME reverse
oKT160	CACTCAACGGTTGGTCAATG	Arabidopsis Real-time PCR GMP forward
oKT161	TGATCTCCTTGTGTGGCAAA	Arabidopsis Real-time PCR GMP reverse
oKT249	CCAAGAAGGAGTGACTCAAGAAAG	Moringa GGP promoter cloning EcoRI cassette 1st reverse
oKT252	CGTGTATAGCCGTGATGTCTACAACT	Moringa GGP promoter cloning XbaI cassette 1st, 2nd reverse
oKT243	GTAATTCGAGACAACAGTGGGAAC	Moringa GGP promoter cloning EcoRI cassette 2nd reverse

Table 2. Identity (%) of amino acid sequences among Moringa and *Arabidopsis* AsA biosynthesis enzymes.

Parameter	GalLDH	GDH	GPP	GGP	GME	GMP
Identity (%)	77	81	81	75	92	86