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Cloning and Characterization of GDP-mannose Pyrophosphorylase from *Solanum Tuberosum* L.

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ABSTRACT : Ascorbic acid is a great antioxidant and helps protect the body against pollutants. GDP-mannose pyrophosphorylase (GMPase) is a key enzyme in manufacturing GDP-mannose, a glycosyl donor for ascorbate and cell wall biosynthesis as well as for protein glycosylation. In this study, we described molecular cloning of a full-length cDNA from Potato (*Solanum tuberosum* L. cv. Jasim), using tuber. The cDNA isolated encoded a GDP-mannose pyrophosphrylase. The nucleotide sequence of pGMPC showed about 95%, 89% and 80% homology with S. *tuberosum* (AF022716), *N. tabacum* (AB066279) and *A. thaliana* (AF076484) cDNAs clone known as GMPase, respectively. We detected the expression of GMPase using RT-PCR. The highest expression of GMPase was found in stems, and the largest amount of ascorbic acid was also presented in stems. In contrast, the leaf showed minimal level of GMPase transcript and ascorbic acid content. We propose that GMPase expression patterns were similar to the changes of ascorbic acid content in the leaves treated with diverse stresses.

Key words : *Solanum tuberosum* L. cv. Jasim, Ascorbic acid, GDP-mannose pyrophosphorylase

INTRODUCTION

L-ascorbic acid (L-AA; vitamin C) is present in milimolar concentrations in plants, where it functions as the major involved in many different processes within the plant cell, including hormone and cell-wall biosynthesis, stress resistance, photoprotection, cell growth (Smirnoff *et al.*, 2000) and possibly other functions still to be discovered.

Humans are unable to synthesize vitamin C, because they have an non-functional gene for L-gulono-1,4-lactone oxidase, which is the last enzyme in the biosynthesis of ascorbate in animals (Nishikimi *et al.*, 1994). Therefore, plant foods are the major source of this essential micronutrient in the human diet, knowledge of the biosynthesis of vitamin C will help to understand the complex role of L-AA and to modify its level in plants (Beata *et al.*, 2001). L-AA has the capacity to directly eliminate several reactive oxygen species, maintain α-tocopherol in the reduced state, and act as a substrate for L-AA peroxidase (Conklin *et al.*, 1999). L-AA is best known for its function as an antioxidant and its role in collagen synthesis. Collagen deficiency results in the symptoms of scurvy. Its role in photosynthesis and photoprotection, in defense against ozone and other oxidative stresses and speculations about its role in cell expansion and cell division will be emphasized (Foyer *et al.*, 1993; Smirnoff *et al.*, 1995). Improved understanding of ascorbate in plants will lead to the possibility of increasing ascorbate concentration in plants by genetic manipulation. This will have benefits for human nutrition and possibly for tolerance of plants to photooxidative stress (Foyer *et al.*, 1993; Smirnoff *et al.*, 1995).

The biosynthesis of vitamin C in plants has been a subject of controversy for many years (Davey *et al.*, 2000). Athough L-galactono-1,4-lactone was recognized as a direct precursor of L-AA, the carbon source for L-galactono-1,4-lactone remained an enigma until recently. The demonstration that D-arabinono-1,4-lactone, the direct precursor of D-erythroascorbic acid in yeast was formed from D-arabinase in a reaction catalyzed by D-arabinase dehydrogenase was undoubtedly of great importance for elucidation of the biosynthesis of vitamin C in plants (Beata *et al.*, 2001). The importance of GDP-mannose pyrophosphorylase in intracellular plant biochemistry is demonstrated by the recent proposal that mannose appears to be involved in the biosynthesis of ascorbic acid (Wheeler *et al.*, 1998). In this pathway, GDP-mannose pyrophosphorylase produces GDP-mannose from mannose-1-phosphate. The GDP-mannose is then converted into GDP-L-galactose by an unusual 3,5-diepimerase. The GDP-L-galactose is subsequently converted into L-galactose-1-phosphate, free L-galacton-1,4-lactone, and

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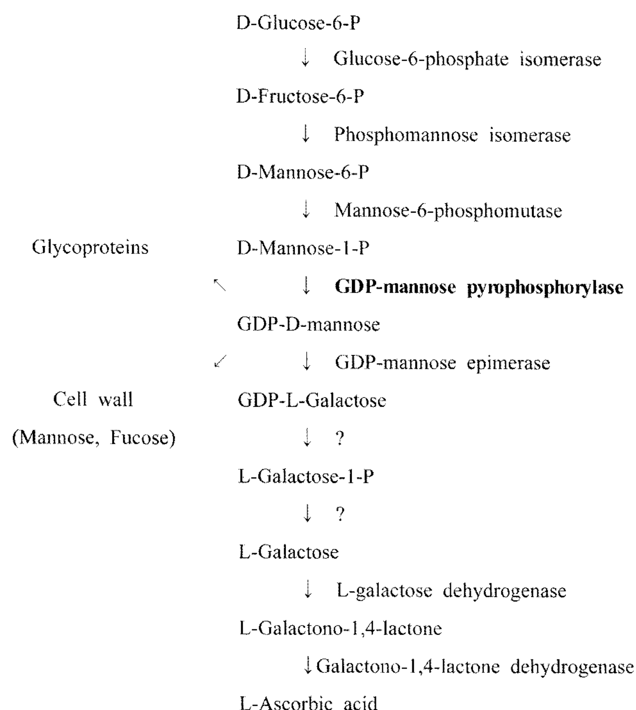

Fig. 1. Proposed pathway for L-AA biosynthesis in higher plants (Wheeler et al., 1998).

finally L-ascorbic acid (Fig.1).

The conversion of radiolabeled mannose into L-galactose is more rate limiting than the conversion of L-galactose into ascorbate. Thus, in addition to its role in glycoprotein biosynthesis, GDP-mannose pyrophosphorylase also appears to play a crucial role in the biosynthesis of vitamin C.

In this study, we carried out to clone GDP- mannose pyrophosphorylase from potato (cv. Jasim), and to determine the detailed gene-characterization studies by bioinformatics. The expression of GDP-mannose pyrophosphorylase and the accumulation of ascorbic acid (L-AA, vitamin C) in response to stress and hormones were also investigated in potato plant tissues.

MATERIALS AND METHODS

Plant materials

Potato plants (*Solanum tuberosum* L. cv. Jasim) were grown in the greenhouse. Samples were taken from the young leaves, stems and tuber. Tissue samples were frozen in liquid nitrogen and kept at −80℃ until use.

Genomic DNA and Total RNA isolation

Potato genomic DNA was extracted by using CTAB method (Park *et al.*, 1998). About 0.1 g of leaf and tuber samples were ground in the mortar at the 700 μl of CTAB buffer (2% cetylrimethylammouium bromide, 0.1 M Tris-HCl, 20 mM EDTA, 1.4 M NaCl, 1% β-mercapto ethanol). The samples were incubated at 60℃ for one hour. Potato total RNA was extracted by LiCl. About 3 g of leaf, steam and tuber samples were ground in the mortar at the 20 μl extract buffer (10 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 8.5 and 0.5% SDS), mixed phenol : chloroform (1 : 1) and centrifuged at 15000 rpm for 15 min. Supernatant were transferred to new tubes and 3 M LiCl were added, mixed well, centrifuged, and supernatant were transferred and two volume of 100% ethanol were added, centrifuged, and washed with 70% ethanol.

Cloning and DNA sequence analysis

Escherichia coli strain DH-5a was used for cloning. Putative GMPase genomic and cDNA sequences were amplified from potato (cv. Jasim) genomic DNA and total RNA using the forward (5'-ATG AAG GCA CTT ATC CTT GTT GG-3') and reverse (5'- TCA CAT CAC GAT TTC AGG TTT CA-3') primers targeted at the 5'- and 3'- end of the open reading frame (ORF) in the GMPase mRNA sequences. Genomic DNA and total RNA were prepared from potato tuber. Genomic polymerase chain reaction (PCR) and reverse transcriptase polymerase chain reaction (RT-PCR) were performed using the TaKaRa Ex Taq (TaKaRa, Japan) and RT-PCR Kit (Promega, USA). The amplification program consisted of 35 cycles of denaturation (94℃, 1 min), annealing (60℃, 1 min) and primer extension (72℃, 1.5 min). Products of the reaction were separated on 1% agarose gels. The purified products were cloned into a pGEM-T easy vector (Promega, USA), and the nucleotide sequences of resulting clones, pGMPG and pGMPC, were determined as described (Roger *et al.*, 1989).

Amino acid sequence alignments

Potato plants (*Solanum tuberosum* L. cv. Jasim) GMPase cDNA and *Solanum tuberosum* GMPase cDNA of nucleotide sequence comparison was performed by information from NCBI (http://www.ncbi.nlm.nih.gov/) using the BLAST program. PROSITE (http://us.expasy.org) program was used to analyze biologically significant sites, patterns and profiles of GMPase. For comparison of amino acid scale defined by a numerical value assigned to each type of amino acid sequence between *S. tuberosum* GMPase and GMPase (*Solanum tuberosum* L. cv. Jasim), PROTSCALE (http://www.expasy.org/cgi-bin/protscale.pl) program was used in Expert Protein Analysis System Molecular Biology Server. This program most frequently used scales are the hydrophobicity or hydrophilicity scales and the secondary structure conformational parameters scales.

Southern blot analysis

Genomic DNA was isolated from tuber tissue. 3 μg of DNA was digested with the restriction enzyme *Bam*H I, *EcoR* I, and *Xho* I, and separated by 1.2% ararose gel electrophoresis, and transferred onto a nylon membrane (Hybond-N+, Amersham, UK). The membrane was prehybridized for 30 min at 65 ℃ in the prehybridization solution containing 5×SSC, 0.1% N-lauroylsarcosine, 0.02% Sodium dodecyl sulfate (SDS), and added 10×blocking (Roche, Germany). The membrane was incubated for 16 h at 65 ℃ after adding the DIG-labeled GMP probe prepared with DIG DNA Labeling Kit (Roche, Germany) in the prehybridization solution. After hybridization the membrane was washed to a final stringency of 0.5×SSC and 0.1% SDS at 65℃ for 2×15 min. DNA on the membrane was detected using the DIG DNA Detection system (Roche, Germany) as described.

Induction conditions

Potato plants (*Solanum tuberosum* L. cv. Jasim) were grown in the greenhouse. Leaves with petioles were spreaded in the solutions for 0, 3, 12, and 24 h : 1.3% H_2O_2 and 100 mM NaCl. For treatments, the leaves were punched with fine pins. For the low temperature, the plant incubated at 4 ℃ for 0, 3, 12, and 24 h. All tissue samples were frozen in liquid nitrogen and stored at −80 ℃ until needed. We detected expression of GMPase used to RT-PCR.

HPLC analysis of ascorbic acid

HPLC analysis of ascorbic acid in plant samples were as previously described (Tarlyn *et al.*, 1998; Keates *et al.*, 2000). Samples were frozen with liquid nitrogen, lyophilized, and stored at −80 ℃. Lyophilized tissues were ground in a chilled mortar and extracted by H_2O containing 6% HPO_3. Ascorbic acid was separated on a μBandapack C_{18} reverse-phase colume (22 ㎝×4.6 ㎝) with H_2O : MeOH (97 : 3) gradient elution at a flow rate 0.7 $\mu\ell$/min. Ascorbic acid was measured by UV at 245 ㎚ (Spectrachrom 100, Thermal Separation Products, San Jose, CA).

RESULTS AND DISCUSSION

Cloning of GMPase

To understand the molecular regulation of GMPase, experiments were designed to isolate the genomic DNA and cDNA for GMPase from potato (cv. Jasim) tuber. Using genomic DNA, mRNA isolated from potato (cv. Jasim) tuber and PCR, RT-PCR in the presence of the primers, each of PCR product was obtained, and sequenced. The genomic DNA and cDNA sequence showing high levels of homology with the known

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  1 atgaaggcacttatccttgtggagggttcggtactcggctcaggc
    M  K  A  L  I  L  V  E  G  S  V  L  G  S  G
 46 acctcaccctcagcgtcccaaagccacttcgtcgaatttgctaac
    T  S  P  S  A  S  Q  S  H  F  V  E  F  A  N
 91 aaaccaatgattttgcatcagattgaggctctcaaggctgttgga
    K  P  M  I  L  H  Q  I  E  A  L  K  A  V  G
136 gtaaccgaagttgtactggctattaactaccaacctgaggtgatg
    V  T  E  V  V  L  A  I  N  Y  Q  P  E  V  M
181 ctgaacttcttgaaagaatttgtcgcaagccttggaatcaagatc
    L  N  F  L  K  E  F  V  A  S  L  G  I  K  I
226 acctgttctcaagaaactgaaccacttggcactgctggtcccctt
    T  C  S  Q  E  T  E  P  L  G  T  A  G  P  L
271 gctttggctagagataagctgataaatgactctggtgaaccatat
    A  L  A  R  D  K  L  I  N  D  S  G  E  P  Y
316 tatgcacttaacagagatgttatcagtcaatatccattcaaggag
    Y  A  L  N  R  D  V  I  S  Q  Y  P  F  K  E
361 atgattcaattccacaaatcccatggtggtgaggcttctttgatg
    M  I  Q  F  H  K  S  H  G  G  E  A  S  L  M
406 gtgaccaaggtggatgagccttctaaatatggtgttgttgtcatg
    V  T  K  V  D  E  P  S  K  Y  G  V  V  V  M
451 gaagaatccactgggcaagtagagagatttgtggagaagccaaag
    E  E  S  T  G  Q  V  E  R  F  V  E  K  P  K
496 ttatttgttggcaacaagatcaatgctggattttacctgctgaac
    L  F  V  G  N  K  I  N  A  G  F  Y  L  L  N
541 ccttctgtcctagacagaattcaattacggccaacatcaattgag
    P  S  V  L  D  R  I  Q  L  R  P  T  S  I  E
586 aaagaggttttccaaaaattgcagcagagaagaaactgtatgca
    K  E  V  F  P  K  I  A  A  E  K  K  L  Y  A
631 atggtgctatctggatttggatggacgttggccaaccaagagat
    M  V  L  S  G  F  W  M  D  V  G  Q  P  R  D
676 ttcattactggcctcagactctatctggactctttaaagaaacac
    F  I  T  G  L  R  L  Y  L  D  S  L  K  K  H
721 tcttcacctaaattggcttcaggaccacacattgtcggaaatgtc
    S  S  P  K  L  A  S  G  P  H  I  V  G  N  V
766 atagtggatgaatctgccaagattggagagggttgtttgatagga
    I  V  D  E  S  A  K  I  G  E  G  C  L  I  G
811 ccagatgttgcaattggttctggttgtgtgattgagtctggagtt
    P  D  V  A  I  G  S  G  C  V  I  E  S  G  V
856 agactctcccgttgcactgtgatgcgaggagtccgcatcaagaaa
    R  L  S  R  C  T  V  M  R  G  V  R  I  K  K
901 catgcatgcatctcaggtagcatcattggctggcactctactgtt
    H  A  C  I  S  G  S  I  I  G  W  H  S  T  V
946 ggacaatggggtcgtggagaacatgaccattctcggggaagatgt
    G  Q  W  G  R  G  E  H  D  H  S  R  G  R  C
991 ccatgtttggtatga 1005
    P  C  L  V  *
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Fig. 2. The Nucleotide sequence and deduced amino acid sequences of GMPase from potato (cv. Jasim).

GMPase sequences were cloned from potato (cv. Jasim). The 1650 bp sequence of the genomic clone, pGMPG, predict the presence of two exons and a 586 bp intron. The presence of intron was confirmed by the nucleotide sequence of the cDNA clone, pGMPC. Comparisons of the nucleotide and deduced amino acid sequences of GMPase with the sequences in the database revealed a high degree of similarity to the plant GMPase. The deduced 10064 nucleotide sequence of the GDP-mannose pyrophorylase shares 95%, 89%, and 80% identity with GDP-mannose pyrophosphorylase from *Solanum tuberosum* (Accession No, AF022716), *Nicotiana. tabacum* (Accession No, AB0662769), and *Arabidopsis. thaliana* (Accession No, AF076484) (Brock *et al.*, 1998). The full length GMPase cDNA is 1064 bp and has one open reading frame of 1005 bp. It is predicted to encode a protein of 334 amino acids (Fig. 2).

The deduced amino acid sequence of potato (cv. Jasim) GMPase cDNA was aligned with that of other plant GMPase genes and showed very close similarity (Fig. 3). The predicted amino acid sequence of potato (cv. Jasim) GMPase shares 89% homology with GMPase of S. *tuberosum* (Accession No, gi1471012), 87% homology with GMPase of N. *tabacum* (Accession No, gi4103323) and 79 % homology with GMPase

of A. *thaliana* (Accession No, gi3598957) (Fig. 3).

All GMPases characterized to date display sequence similarity in their catalytic domains. The overall features of this organization are present in the catalytic domain of the GMPase protein since all the absolutely conserved amino acids as well as the highly conserved amino acid groups were identified.

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GNP_N.   MKALILV-GG-FGTRLRPLTLSVPKPLVEFANKPNILHQIEALKAVGVTEVVLAINVQPE
GNP_A.   MKALILV-GG-FGTRLRPLTLSFPKPLVDFANKPNILHQIEALKAVGVDEVVLAINVQPE
GNP_S.   MKALILV-GG-FGTRLRPLTLSVPKPLVEFANKPNILHQIEALKAVGVTEVVLAINVQPE
GNP      MKALILVEGSVLGSGTSP-SAS-QSHFVEFANKPNILHQIEALKAVGVTEVVLAINVQPE
         ******* *, :*:   * : *   . :*:******************** ***********

GNP_N.   EMLNFLKEFEANLGIKITCSQETEPLGTAGPLALARDKLIDDSGEPFFVLNSDVISEVPF
GNP_A.   VMLNFLKDFETKLEIKITCSQETEPLGTAGPLALARDKLLDGSGEPFFVLNSDVISEVPL
GNP_S.   VMLNFLKEFEASLGIKITCSQETEPLGTAGPLALARDKLIDDSGEPFFVLNSDVISEVPF
GNP      VMLNFLKEFVASLGIKITCSQETEPLGTAGPLALARDKLINDSGEPVVALNRDVISQVPF
          ******:* :.* ************************::.****::.** ****:**:

GNP_N.   KEMIAFHKSHGGEASLMVTKVDEPSKYGVVVMEESTGQVERFVEKPKLFVGNKINAGFVL
GNP_A.   KEMLEFHKSHGGEASIMVTKVDEPSKYGVVVMEESTGRVEKFVEKPKLVVGNKINAGIVL
GNP_S.   KEMIQFHKSHGGEASLMVTKVDEPSKYGVVVMEESTGQVERFVEKPKLFVGNKINAGFVL
GNP      KEMIQFHKSHGGEASLMVTKVDEPSKYGVVVMEESTGQVERFVEKPKLFVGNKINAGFVL
         ***: **********:************************:**:********:*********:**

GNP_N.   LNPSVLDRIQLRPTSIEKEVFPKIAAEEKLYANVLPGFWMDIGQPRDYIPGLRLYLDSLK
GNP_A.   LNPSVLDKIELRPTSIEKETFPKIAAAQGLYANVLPGFWMDIGQPRDYITGLRLYLDSLR
GNP_S.   LNPSVLDRIQLRPTSIEKEVFPKIAAEKKLYANVLPGFWMDIGQPRDYITGLRLYLDSLK
GNP      LNPSVLDRIQLRPTSIEKEVFPKIAAEKKLYANVLSGFWMDVGQPRDFITGLRLYLDSLK
         *******:*:**********.****** : ******.*****:*****:*.*********:

GNP_N.   KNSSP-KLASGSHIVGNVIVDESAKIGEGCLIGPDVAIGSGCVIESGVRLSRCTVMRGVR
GNP_A.   KKS-PAKLTSGPHIVGNVLVDETATIGEGCLIGPDVAIGPGCIVESGVRLSRCTVMRGVR
GNP_S.   KHSSP-KLASGPHIVGNVIVDESAKIGEGCLIGPDVAIGSGCVIESGVRLSRCTVMRGVR
GNP      KHSSP-KLASGPHIVGNVIVDESAKIGEGCLIGPDVAIGSGCVIESGVRLSRCTVMRGVR
         *:* * **:**.******:***:*.**************.**::****************

GNP_N.   IKKHACISSSIIGWHSTVGQWARVENMTILGEDVHVCDEIYSNGGVVLPHKEIKSSILKP
GNP_A.   IKKHACISSSIIGWHSTVGQWARIENMTILGEDVHVSDEIYSNGGVVLPHKEIKSNILKP
GNP_S.   IKKHACISGSIIGWHSTVGQWARVENMTILGEDVHVCDEIYSNGGVVLPHKEIKSSILKP
GNP      IKKHACISGSIIGWHSTVGQWGR-------GE--H--D--HSRGRC--P-----C--L--
         ********.************.*       **  *  *  :*.*    *     .  *

GMP_N.   EIVM
GMP_A.   EIVM
GMP_S.   EIVM
GMP      --V-
           *
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Fig. 3. Alignment and comparison of deduced amino acid sequence of *S. tuberosum* cv. Jasim GMPase (GMP) with GMPase sequence of *S. tuberosum* (GMP_S), *N. tabacum* (GMP_N) and *A. thaliana* (GMP_A).

The hydropathy and polarity index plot of GMPase (A) compared to GMPase of S. *tuberosum* (B) revealed. The hydropathy profile of the deduced GMPase amino acid sequences (Fig. 4 A) revealed the presence of several hydrophobic regions, four of which were predicted by the TOPRED2 program to be transmembrane domains. These putative membrane-spanning domains are located between residues 2 and 29,32 and 64, 263 and 292, and 296 and 321. Each of which is long enough to span a membrane bilayer, these are conserved in other GMPase characterized in plant.

The hydropathy profile of GMPase, deduced by the algorithm of Kyte and Doolittle (Kyto *et al.*, 1982) showed close similarity to the GMPase of S. *tuberosum*, but the degree of hydrophobicity of GMPase revealed higher than GMPase of S. *tuberosum* (Fig. 4). This result mean that amino acid sequence of GMPase expressed higher polarity than that of GMPase of S. *tuberosum* (Fig. 5). Positive value indicated that free energy was required for transfer to water, and it was characterized degree of hydrophobicity.

The pro-site composed of N-glycosylation site, cAMP-and cGMP-dependent protein kinase phosphorylation site, Protein kinase C phosphorylation site, Casein kinase II phosphorylation site, Tyrosine kinase phosphorylation site, and N-myristoylation site.

N-glycosylation site is located at position 99 to 102

(A) (B)

Fig. 4. Hydropathy index plot of the proedicted amino acid residues. (*S. tuberosum* (cv. Jasim) GMPase (A), *S. tuberosum* GMPase (B).)

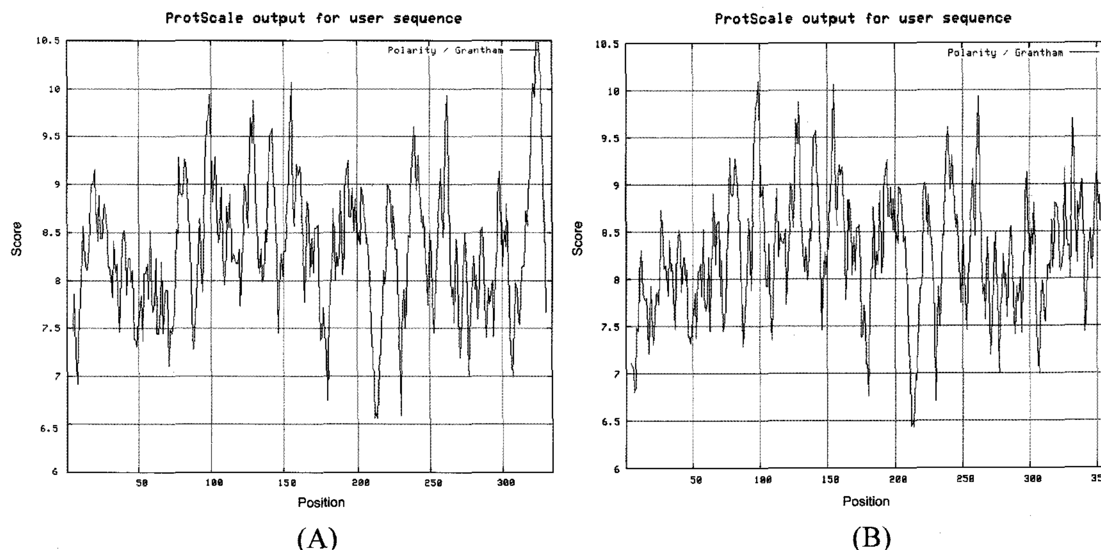

Fig. 5. Comparison of polarity index of the complete amino acid sequence of GMP by PROSITE. (*S. tuberosum* (cv. Jasim) GMPase (A), *S. tuberosum* GMPase (B).)

(NDSG). Protein kinase C phosphorylation site is located at position 236 to 238 (SIK), 242 to 244 (SpK) and 260 to 262 (SaK). Casein kinase II phosphorylation site is located at position 137 to 140 (TkvD), 182 to 185 (SvID) and 192 to 195 (TsiE). Tyrosine kinase phosphorylation site is located at position 138 to 145 (KvdEpskY) and 201 to 209 (KiaaEkkIY). N-myristoylation site is located at position 9 to 14 (GSvIGS), 15 to 20 (GTspSA), 72 to 77 (GIkiTC), 129 to 134 (GGeaSL), 169 to 174 (GNkiNA), 284 to 289 (GVrISR) and 306 to 311 (GSiiGW). Finally active- site is AMP-and cGMP-dependent protein kinase phosphorylation site is located at position 238 to 241 (KKhS).

Genomic Southern blot analysis

The copy number of the GMPase gene in potato (cv. Jasim) genome was estimated by genomic southern blot analysis. A southern blot assay carried out genomic DNA digested with restriction endonucleases *Bam*H I, *EcoR* I, and *Xho* I, was hybridized with DIG-labeled GMP probe. Two bands were observed in line 1 (Fig. 6). Several bands were observed in the DNA digested with *EcoR* I and *Xho* I. These endonuclease sites are not present in the sequence used to probe the blot, suggesting that more than one copy of GMPase-related genes are present in the potato genome.

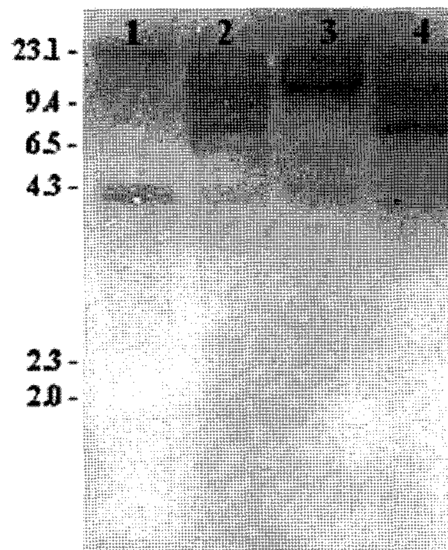

Fig. 6. Genomic Southern blot hybridization analysis to verify the number of GMPase in potato (cv. Jasim). Potato genomic DNA was digested with *Bam*H I (line 1), *EcoR* I (line 2), *Xho* I (line 3), and *EcoR* I + *Xho* I (line 4). DNA molecular size markers are indicated on the left.

Transcript analysis of Potato GMPase from different organs

Distribution of ascorbic acid in various tissues of Potato plants grown in the greenhouse was also determined, and expression of GMPase was detected in all tissues. However, there were differences in the levels of the GMPase transcripts. The maximal level of expression was found in stems (Fig. 7), and the largest amount of ascorbic acid (66.9 $\mu g/\mu \ell$) was also present in stems. In contrast, the minimal level of expression was found in leaves. A trace amount of ascorbic acid (56.7 $\mu g/\mu \ell$) was present in leaves. The difference in the intensity of the GMPase signal in the another sample is thought to be ascorbic acid transport system. Loewus *et al.*, (1987) published that photosynthetic organs, certain storage organs, and

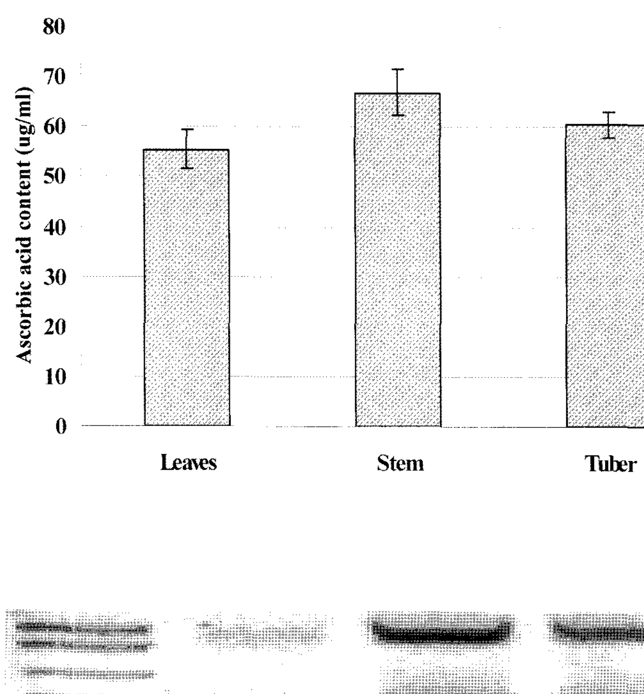

Fig. 7. Accumulation of ascorbic acid (A), and analysis of Potato GMPase transcripts (B). A. ascorbic acid content was determined by HPLC. B. Total RNA was isolated from different organs. Frist-strand cDNA was synthesised and used as the template for RT-PCR.

meristeme are known to have high concentrations of ascorbic acid and there is a consistent theme in the literature of highest ascorbic acid levels in non-photosynthetic organs during their most rapid phase of growth. This includes such diverse fruits as mango (Kudachikar *et al.*, 2001), peppers (Yahia *et al.*, 2001), and other organs such as stolon tips (Viola *et al.*, 1998), developing lateral roots (Innocenti *et al.*, 1993), and germinating embryo axis (Pallanca *et al.*, 2000). This tissues specific distribution of ascorbic acid and GMPase mRNA may suggest spatial regulation of GMPase gene expression and ascorbic acid synthesis in potato plants.

Accumlation of ascorbic acid and GMPase mRNA in response to several stresses

A variety of undesirable environmental conditions can lead an excessive production of reactive oxygen species (ROS, e.g. O^-_2, H_2O_2 and OH) in plant cells, resulting in a condition called oxidative stress and causing damage to proteins, membranes and other cell constituents. In response, plants can quickly increase the activities of their antioxidant genes to defend themselves. It has been shown that drought (Jahnke *et al.*, 1991), high intensity light (Inze *et al.*, 1995), low temperature (Hendry *et al.*, 1994), and ozone (Graham *et al.*, 1998) can all induce ascorbate peroxidase. Recent years have witnessed a plethora of reports correlating increases in one or more of the antioxidant enzymes with either stress conditions or ameliorated stress resistance. Abiotic conditions that have been studied include, among others, low temperature (Hendry *et al.*, 1994), high salinity (Inze *et al.*, 1995), herbicide challenge (Gillham *et al.*, 1987), drought, wounding, ultraviolet irradiation (Jahnke *et al.*, 1991), SO_2 fumigation (Kubo *et al.*, 1995), and ozone exposure (Graham *et al.*, 1998). In work where several enzymes have been studied under the same stress conditions, differential responses have frequently been observed.

In this paper, leaves were spreaded the following sterile solutions, 50 mM sodium chloride, for 0, 3, 12 and 24 h. For the wounding treatment, leaves were punched with fine pins. For the low temperature, the plant incubated at 4 ℃, and total RNA and ascorbic acid isolated from each samples.

As shown in Fig. 8A, ascorbic acid decreased for the 3 h, and then increased. However, ascorbic acid decreased after low temperature. GMPase expression patterns were similar to the changes in ascorbic acid content in the leaves treated low temperature. GMPase transcript increased for the 3 h, and then decreased. This result indicate that GMPase expression patterns were similar to the changes in ascorbic acid level in the leaves treated with 50 mM sodium chloride. However, ascorbic acid decreased to 2-fold after 24 h treatment (Fig. 8B). GMPase transcript decreased for 12 h, and then increased. This result indicates that its expression patterns were similar to the changes in ascorbic acid content in the leaves treated with wounding (Fig. 8C). Ascorbic acid decreased about 2-fold 12 h after the wounding treatment, and they progressively increased from 24 h after the wounding treatment. A similar result was shown in white potato tuber tissue (Oba *et al.*, 1994). The ascorbic acid content decreased slightly during the first 12 h and then increased by 40% within 36 h after treatment, and slowly decreased thereafter. The level of dehydro ascorbic acid (DHA) was low initially, being only 2 μg ; at 36 h it started to increase and reached 10 μg at 48 h after white potato tuber treated wounding.

Leaves were spreaded the following sterile 1% H_2O_2 solutions for 0, 3, 12 and 24 h. After H_2O_2 treatment, total RNA from each sample isolated and ascorbic acid and monodehydroascorbate content measured.

As shown in Fig. 8D, ascorbic acid content was decreased to 6-fold 24 h after H_2O_2 treatment. However, monodehydroascorbate content increased for the 12 h treatment, and then decreased. GMPase transcript increased for the 3 h treatment, and then decreased.

Monodehydroascorbate content was similar to the ascorbic

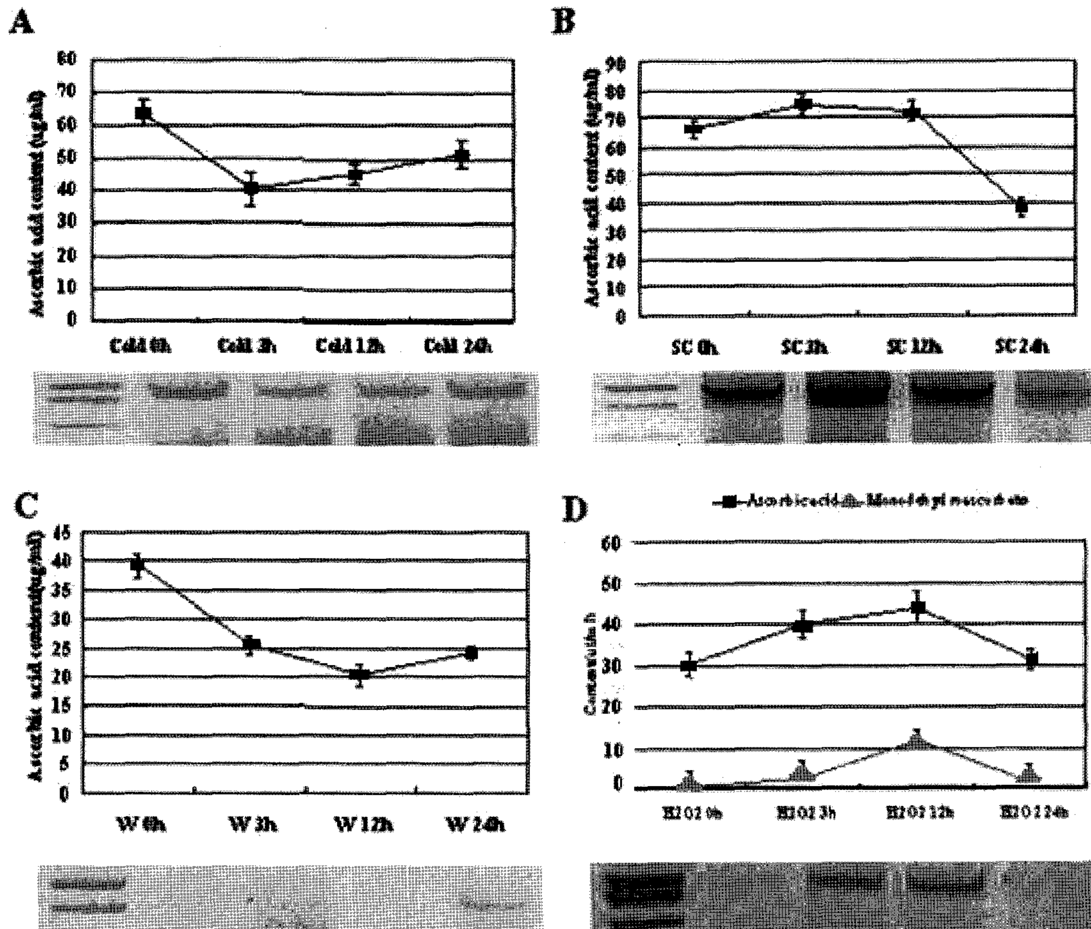

Fig. 8. Accumulation of ascorbic acid, and analysis of Potato GMPase transcripts. A. The plant incubated at 4℃, B. Leaves were spreaded 50 mM sodium chloride, C. leaves were punched with fine pins, D. Leaves were spreaded the following sterile 1% H_2O_2.

acid content, after 12 h treatment.

The ascorbate-glutathione cycle is the major antioxidant defense system pathways. Superoxide radicals, O_2 are eliminated by superoxide dismutase in a reaction that yields hydrogen peroxide, H_2O_2. Hydrogen peroxide is consumed through its conversion to oxygen and water by catalase or to water alone through the oxidation of ascorbate. Ascorbate is regenerated by the way of two mechanisms. The enzymatic reduction of monodehydroascorbate takes place in the plastids. Alternatively, monodehydroascorbate that is spontaneously dismutated to dehydroascorbate can react with glutathione (GSH) to produce ascorbate and oxidized glutathione (GSSG) in a reaction catalyzed by dehydroascorbate reductase. GSSG is reduced by glutathione redutase, requiring the consumption of NADPH. Singlet oxygen and hydroxyl ions are eliminated in the glutathione pathway. Damage by singlet oxygen and hydroxyl ions is also diminished by the nonenzymatic antioxidants, vitamin E and carotenoids (Bob *et al*., 2000).

Ascorbate peroxidase (APX) uses ascorbate as an electron donor to scavenge H_2O_2 and other organic hydroperoxides produced during normal cellular metabolism (Asada *et al*., 1992). The reactions catalysed by APX is : 2 ascorbate + H_2O_2 (ROOH) $\rightarrow$ 2 monodehydroascorbate + $2H_2O_2$ (ROH+H_2O)

We could observe that leaves in the H_2O_2 treatment changed ascorbic acid into monodehydroascorbate.

In this study, we have isolated the gene encoding GDP-mannose pryophosphorylase (GMPase). Furthermore, we proposed that GMPase expression patterns were similar to the changes in ascorbic acid content in the leaves treated with diverse stresses. Ascorbic acid controls cell division/elongation and also play an important role in photoprotection system. However, the detailed mechanism controlling ascorbic acid levels in cells remains unclear. Further analysis of the relationship between ascorbic acid level and ascorbic acid-related enzyme will be required to clarify regulation mechanism of ascorbic acid in plants.

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LITERATURE CITED

Asada K (1992) Ascorbate peroxidase-a hydrogen peroxide scavenging enzyme in plants. Physiol. Plant. 85:235-241.

Beata AW, Geert P, Doorsselaere JV, Mart WD, Boerjan W (2001) Partial purification and identification of GDP- mannose 3", 5"-epimerase of *Arabidopsis thaliana*, a key enzyme of the plant vitamin C pathway. J. Biol. Chem. 98:14843-14848.

Bob B, Buchanan WG, Russell LJ (2000) Biochemistry and Molecular Biology of plants. Chapter 22. Responses to abiotic stresses.

Brock , Robert T (1998) Characterization of the cDNA and Gene for the Arabidopsis thaliana GDP-mannose pyrophosphorylase (Accession No. AF076484) (PGR98-175). Plant Physiology 118:1101.

Conklin P L, Norris SR, Wheeler GL, Williams EH, Smirnoff N, Last RL (1999) Genetic evidence for the role of GDP-mannose in plant ascorbic acid(vitamin C) biosynthesis. Proc. Natl. Acad. Sci. USA. 96:4198-4203.

Davey MW, Van MM, Inze D, Sanmartin M, Kanellis A, Smirinoff N, Fletcher J (2000) J. Sci. Food Agric. 80:825-860.

Foyer CH, Lelandais M (1993) The roles of ascorbate in the regulation of photosynthesis. In : Yamamoto HY, Smith CM, eds. photosynthetic responses to the environment. Rockville, Maryland : Amer. Soc. Plant Physiologists 88-101.

Gillham DJ, Dodge AD (1987) Chloroplast superoxide and hydrogen peroxide scavenging systems from pea leaves: seasonal variation. Plant Sci. 50:105-109.

Graham N, Chistine HF (1998) Ascorbate and Glutathione : Keeping active oxygen under control. Annu. Rev. Plant Physiol. Plant Mol. 49:249-279.

Hendry GAF, Crawford RMM (1994) Oxygen and environmental stress in plant: an overview. Pro Royal Soc Edinburgh. 102B:1-10.

Innocenti AM, Bitonti MB, Mazzuca A, Liso R, Arrigoni O (1993) Histochemical localization of exogenous ascorbic acid in the pericycle nuclei of Vicia faba. Caryologia 46:1-4.

Inze D, Van Montagu M (1995) Oxidative stress in plants. Curr Opin Biotechnol. 6: 153-158.

Jahnke LS, Hull MR, Long SP (1991) Chilling stress and oxygen metabolizing enzymes in Zea mays and Zea diploperennis. Plant Cell Environ. 14:97-104.

Keates SE, Tarlyn NM, Loewus FA, Franceschi VR (2000) L-Ascorbic acid and L-galactose are sources for oxalic acid and calcium oxalate in Pistia stratiotes. Phytochemistry 53:433-440.

Kubo A, Saji H, Tanaka K,nKondo N (1995) Expression of Arabidopsis cytosolic ascorbate peroxidase gene in response to ozone or sulfur dioxide. Plant Mol Biol. 29:479-489.

Kudachikar VB, Kulkarni SG, Prakash MNK, Vasantha MS, Prasad BA, Ramana KVR (2001) Physio-chemical changes during maturity of mango (*Mangifera indica* L.) variety "Neelum." J Food Sci Technol. 38:540-542.

Kyto J, Doolittle RF (1982) A sample method of displaying the hydropathic character of a protein. J. Mol. Biol. 157:105-132.

Loewus FA, Loewuns MW (1987) Biosynthesis and metabolism of ascorbic acid in plants. CRC Crit. Rev. Plant Sci. 5:101-119.

Nishikimi M, Fukuyama R, Minoshima S, Shimizu N, Yagi K (1994) Cloning and chromosomal mapping of the human nonfunctional gene for L- galactono-gamma-lactone oxidase, the enzyme for L-ascorbic acid biosynthesis missing in man J. Biol. Chem. 269:13685-13688.

Oba K, Fukui M, Imal Y, Iriyama S, Nogami K (1994) L-galactono-gamma-lactone dehydrogenase - partial characterization, induction of activity and role in the synthesis of ascorbic acid in wounded white potato-tuber tissue. Plant and Cell Physiology 35(3):473-478.

Pallanca J E, Smirnoff N (2000) The control of ascorbic acid synthesis and turnover in pea seedlings. J. Exp. Bot. 51:669-674.

Park CH, Seong NS, Paek KY, Lee CH (1998) Micropropagation through callus culture in Chinese foxglove (*Rehmannia glutinosa*). Korean J. Plant Tissue Culture 25(3):171-175.

Roger SO (1989) Bendich extraction of DNA from plant tissues ; in plant molecular biology manual, Gelvin SB, Schilperoort RA, Verma DPS (eds), A6: 1-10.

Smirnoff N (1995) Antioxidant systems and responses to the environment. In : Smirnoff N ed. Environment and plant metabolism. Flexibility and acclimation. Oxford: Bios Scientific Publishers. 217-243.

Smirnoff N, Wheeler GL (2000) Ascorbic acid in plant : biosynthesis and function. Crit. Rev. Plant Sci. 19:267-290.

Tarlyn NM, Kostman TA, Nakata PA, Keates SE, Franceschi VR (1998) Axenic culture of Pistia stratiotes for use in plant biochemical studies. Aquatic Bot. 60:161-168.

Viola R, Vreugdenhil D, Davies HV, Sommerville L (1998) Accumulation of L-ascorbic acid in tuberising stolon tips of potato (*Solanum tuberosum L.*). J Plant Physiol. 152:58-63.

Wheeler GL, Jones MA, Smirnoff N (1998) The biosynthetic pathway of vitamin C in higher plants. Nature 393:365-369.

Yahia EM, Contreras-Padilla M, Gonzalez-Aguilar G (2001) Ascorbic acid content in relation ascorbic acid oxidase activity and polyamine content in tomato and bell pepper fruits during development, maturation and senescence. Food Sci Technol. 34: 452-457.