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Molecular Identification of Two Genes of Germin-like Protein in *Arabidopsis*

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Abstract

We identified two homolog genes of *PnGLP* (*Pharbitis nil* germin-like protein) and *SaGLP* (*Sinapis alba* GLP) in *Arabidpsis thaliana*, namely, *AtGLP1* and *AtGLP2*. Results of Southern blotting showed that each gene was single per haploid genome. Northern blotting revealed the presence of mRNAs for both genes in all overground parts, but especially in the leaf and flower. RFLP mapping showed that *AtGLP1* was on chromosome 1 (112.4±0.6 cM) and *AtGLP2* was on chromosome 5 (35.7±0.3 cM).

1. Introduction

Germins and germin-like proteins (GLPs) constitute a large family of genes in plants. Germin was initially determined in germinating wheat seedings as a molecular marker for early germination [1]. The amino acid sequence of germin has similarity to an amoeba protein [2]. The structure of sugar residues of germin has been determined [3]. Although germin has enzymatic activity of oxalate oxidase (EC. 4.3.2.1) [4], its physiological function remains unknown. Recently, several cDNAs encoding germin and GLPs have been isolated from various plant species of various organs. *McGLP* was isolated from a halophyte *Mesembryanthemum crystallinum* (common ice plant) as a root specific cDNA [5]. ATTS0248 was isolated from *Arabidopisis* from a developing silique mRNA as an expressed sequence tag (EST). *Pharbitis nil* GLP (*PnGLP*) was determined as a leaf-specific protein from the short-day induced cotyledons [6, 7]. *Sinapis alba* GLP (*SaGLP*) was determined as a long-day inducible leaf cDNA [8]. These GLPs did not have detectable oxalate oxidase activity, and their physiological roles were not known. We previously discussed that germins and GLPs were divided into several groups. We called *PnGLP and SaGLP* leaf-specific GLPs [7], because *PnGLP* and *SaGLP* shared several distinct features from other GLPs: circadian rhythmic expression, leaf specific expression and dissimilarity in amino-acid sequences.

We have been studying the short-day plant *Pharbitis nil* cv Violet, which can be induced to flower by a single short-day treatment even in the seedling stage. We analyzed changes in several macro molecules in cotyledons during photoperiodic induction using two-dimensional gel electrophoresis [6, 9, 10]. In the experiments on *in vivo* labelled cotyledonous protein, we found the 22 kDa protein spot that showed a specific increase with flower inductive short-day treatments [6]. We isolated and characterized the cDNA of the 22 kDa protein, and named it *PnGLP*. The mRNA for *PnGLP* was leaf-specific and steady-state level of *PnGLP* mRNA showed a transient increase during the inductive darkness [7]. On the other hand, cDNA of *SaGLP* has been isolated from *Sinapis alba* a long-day plant. The cDNA of *SaGLP* was determined by differential screening of cDNA libraries to isolate long-day specific cDNAs [8]. Interestingly, the steady-state levels of *SaGLP* mRNA also showed fluctuation with the light/dark cycles. Therefore, we speculated that *PnGLP* and *SaGLP* relate to photoperiodic events including flower induction [7]. Because *Pharbitis* is not suitable for molecular and genetic studies, we decided to use *Arabidopsis* for these studies on leaf-specific GLPs. We reported here genomic organization, differential mRNA accumulation and map position of *Arabidopsis* homologs of *PnGLP* and *SaGLP*, *AtGLP1* and *AtGLP2*.

2. Materials and Methods

2.1 Plant materials

Seeds of *Arabidopsis thaliana* ecotype Columbia wild (kindly provided by Dr. Y. Komeda of Hokkaido University) were sown on soil (Kureha Engei-Baido: Kureha Chemical Co., Tokyo, Japan) in plastic pots and were placed in a cold room at 4°C for 3 days before being transferred to a growth chamber operating at 22°C under cool-white fluorescent light (FL40SS, FL20SS and FL15W: Toshiba Electronics

Co., Tokyo, Japan) with an 8-h light and 16-h dark photoperiod. Three-month-old plants bearing flowers and developing siliques were used. Leaf, cauline leaf, stem, root, flower and developing siliques were harvested and frozen in liquid nitrogen and stored at −80°C until RNA isolation. Total RNA was isolated as described by Nagy *et al.* [11].

2.2 *Isolation of homolog of* PnGLP *and* SaGLP *in* Arabidopsis

Two oligonucleotide primers were designed for polymerase chain reaction (PCR): primer CGLS 5′-GCNWSNGTNMANGAYTTYTGYGT-3′ and primer CGLA 5′-ARNARNCCYTGNGGRAANACCAT-3′ (where N=A, C, G, or T; W=A or T; S=C or G; M=A or C; Y=C or T; R=A or G). The reaction mixture for PCR contained 1 μg of the genomic DNA, GeneAmp PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001% (w/v) gelatin], primer CGLS and CGLA each at 20 μM, 200 μM dNTPs, and 2.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, U.S.A.). PCR was performed in a total volume of 50 μl in a thermal cycler with four successive steps: denaturation (10 min at 95°C); three initial cycles (1 min at 95°C, 1 min at 57°C and 1 min at 72°C after gradual heating from 57°C to 72°C for 3 min); 22 successive cycles (1 min at 95°C, 1 min at 57°C and 1 min at 72°C; and a final incubation for 5 min at 72°C. DNA fragments of 386-bp were obtained, cloned into pCRII (Invitrogen Co., San Diego, CA, U.S.A.), and DNA sequenced. Nucleotide and deduced amino-acid sequences were analyzed with GENETYX-MAC software, version 8.0 (Software development Co., Tokyo, Japan). Databases were searched with the DDBJ BLAST system [DNA Data Bank of Japan; Mishima, Shizuoka, Japan; 12]. Furthermore, these DNA fragments (386 bp) were used to screen a genomic library of *Arabidopsis thaliana* ecotype Columbia in λ EMBL3 [13] (Clontech Lab. Inc., Palo Alto, CA, U.S.A.). Several genomic clones containing *AtGLP1* or *AtGLP2* were purified, subcloned into pBlueScript plasmids and DNA sequenced.

2.3 *DNA gel blot hybridization*

Genomic DNA was isolated from seedlings as described by Rogers and Bendich [14] and it was digested with *Eco*RI, *Bam*HI, *Hin*dIII and *Xba*I. Digested DNA was subjected to electrophoresis on an agarose gel, and bands of DNA were transferred to a nylon membrane filter (Biodyne B; Nihon Pall Ltd., Tokyo, Japan). The DNA on the filter was allowed to hybridize with ^{32}P-labelled 386-bp DNA fragments of *AtGLP1* in a hybridization solution that contained 6x SSPE (1x SSPE is 0.18 M NaCl, 0.01 M sodium phosphate, and 1 mM Na_2 EDTA, pH 7.7), 5x Denhardt's solution (1x Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 0.5% SDS, and 150 μg/ml of salmon sperm DNA at 65°C for 16 h. The filter was washed twice with 2x SSPE and 0.1% SDS for 5 min at room temperature, and then twice for 30 min at 65°C (low-stringency conditions). After exposure to an imaging plate for an appropriate time, the same filter was washed twice with 0.1x SSPE and 0.1% SDS for 30 min at 65°C (high-stringency conditions). For visualization of bands on the filter, we used a bio-imaging analyzer with an imaging plate (BAS2000; Fuji Photo Film Co., Tokyo, Japan). After removal of *AtGLP1* probe DNA, the same filter was reprobed with ^{32}P-labelled 386-bp DNA fragments of *AtGLP2*.

2.4 *RNA gel blot hybridization*

Total RNA (20 μg) was fractionated by electrophoresis on a formaldehyde-agarose gel and the bands of RNA were transferred to a nylon membrane filter (Biodyne B). The RNA on the filter was allowed to hybridize with ^{32}P-labelled 386-bp DNA fragments of *AtGLP1* in a hybridization solution that contained 50% formamide, 5x SSPE, 5x Denhardt's solution, 0.1% SDS, and 150 μg/ml salmon sperm DNA at 42°C for 20 h. The filter was first washed with 2x SSC at room temperature and then with 2x SSC and 0.1% SDS at 42°C. For visualization of the bands on filter, we again used the bio-imaging analyzer. After removal of *AtGLP1* probe DNA, the same filter was reprobed with ^{32}P-labelled 386-bp DNA fragments of *AtGLP2*.

2.5 *RFLP mapping*

The 100 recombinant inbred (RI) lines from a cross between *Arabidopsis thaliana* ecotype Columbia and Landsberg *erecta* [15; kindly provided by the Nottingham Arabidopsis Stock Centre] were used for mapping *AtGLP1* and *AtGLP2*. A 7-kbp fragment of *Sal*I digested genomic clone containing entire coding sequence of *AtGLP1* [Sage-Ono *et al.*, unpublished results] and the PCR-amplified fragment of *AtGLP2* (386 bp) were used as a probe for RFLP (restriction fragment length polymorphism) mapping. The restriction endonucleases that showed RFLP between Columbia and Landsberg *erecta* in genomic DNA fragments containing *AtGLP1* or *AtGLP2* were used for RFLP mapping. For *AtGLP1*, *Dra*I was used to generate mapping blots containing *Dra*I-digested DNA from 100 RI lines. For *AtGLP2*, *Xba*I was used. Linkage analysis was performed using the MAPMAKER mapping program of Lander *et al.*, [16]. We used the Macintosh version 2.0, which differed from version 1.0 in allowing the recombina-

tion frequency to be calculated using an algorithm appropriate for recombinant inbred lines. Our criterion for establishing linkage between loci was a minimum LOD score of 6.0. MAPMAKER's Kosambi mapping function option was used to convert recombination frequencies to centimorgan.

3. Results

3.1 *Isolation of homologs of* PnGLP *and* SaGLP *in* Arabidopsis

Previously, we reported high sequence similarity of *PnGLP* and *SaGLP*. The amino acid sequence of *PnGLP* was 55% identical to that of *SaGLP* [7]. We made a pair of degenerate DNA primers for PCR amplification for isolation of homologs of *PnGLP* and *SaGLP* from other plants. These primers corresponded to ASVN/QDFCVA (CGLS: from 22 to 30 amino acid of *PnGLP*) and MVFPQGLL (CGLA: from 144 to 151 amino acid of *PnGLP*). The PCR amplification using genomic DNA of *Arabidopsis* as a template generated 386-bp DNA fragments. These DNAs were cloned into pCRII plasmid and were determined their DNA sequence. Results were classified into two sequences. We found that both DNA fragments encoded similar GLP proteins and named them *AtGLP1* and *AtGLP2*. These nucleotide sequences were deposited to the DDBJ, EMBL and GenBank nucleotide sequence databases under accession numbers D89055 (*AtGLP1*) and D89374 (*AtGLP2*). Results of database searching revealed that there are many ESTs identical to *AtGLP1* and *AtGLP2* and there are no other transcriptable genes having significant homology to *PnGLP* and *SaGLP*. We isolated corresponding genomic DNA clones [Sage-Ono *et al.* unpublished results] from a genomic library of ecotype Columbia constructed in λ phage EMBL3. The coding sequences were used for deducing their amino acid sequences. The amino acid sequence of *AtGLP1* exhibits 64%, 59%, and 65% identity to *AtGLP2*, *PnGLP* and *SaGLP*, respectively. The amino acid sequence of *AtGLP2* exhibits 54% and 94% identity to *PnGLP* and *SaGLP*, respectively. We made a molecular phylogenic tree of amino acid sequence of germins and GLPs for comparison (**Fig. 1**). The tree clearly indicated the distinctive position of leaf-specific GLPs, namely, PnGLP, SaGLP, AtGLP1 and AtGLP2, against other GLPs.

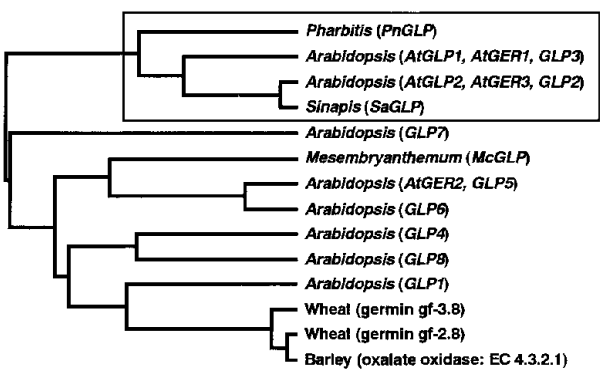

Fig. 1 A molecular phylogenic tree of the amino acid sequence of germins and germin-like proteins. The leaf-specific germin-like proteins are boxed. The tree was constructed by the UPGMA method using GENETYX-MAC software (Software Development Co., Ltd., Tokyo, Japan). The abbreviations and sources of the amino acid sequences used for these comparisons are as follows: *PnGLP* (*DDBJ accession number D45425*), *AtGLP1* (D89055), *AtGER1* (Z30804), *AtGLP2* (D89374), *AtGER3* (Z26437 and Y12673), *SaGLP* (X84786), *GLP1* to 8 (U75187-75207), *McGLP* (M93041), wheat germin gf-3.8 (A40391), germin gf-2.8 (B40391) and barley oxalate oxidase (L15737).

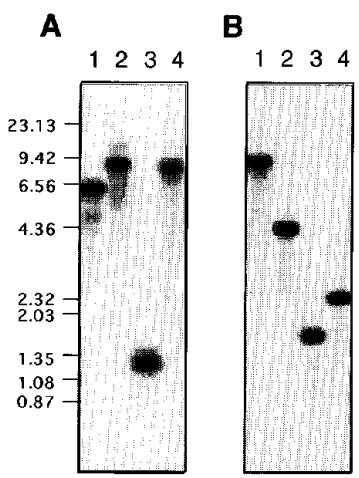

Fig. 2 DNA gel blot analysis of *AtGLP1* and *AtGLP2*.

Genomic DNA was prepared from *Arabidopsis thaliana* (ecotype Columbia wild), digested with *Eco*RI (lane 1), *Bam*HI (lane 2), *Hin*dIII (lane 3) and *Xba*I (lane 4), and subjected to DNA gel blot hybridization. Results of low stringency washing are presented. (A) Results of *AtGLP1* probed with a 386-bp DNA fragments of *AtGLP1*. (B) Results of *AtGLP2* probed with a 386-bp DNA fragments of *AtGLP2*.

Numbers at left indicate molecular length markers in kilobases.

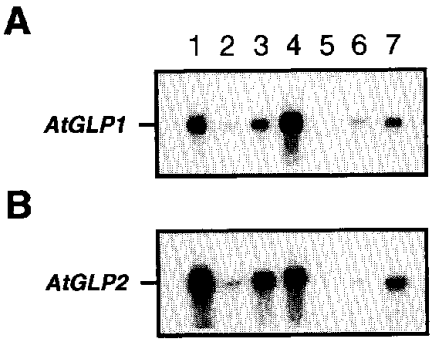

Fig. 3 RNA gel blot analysis of *AtGLP1* and *AtGLP2* in different organs.
Total RNA was isolated from various organs of seedlings of *Arabidopsis thaliana* ecotype Columbia wild [lane 1: flower, 2: silique, 3: stem, 4: cauline leaf, 5: root, 6: leaf at 'Zeitgeber' time (zt)4, and 7: leaf at zt16]. Total RNA (20 μg per lane) was fractionated by gel electrophoresis.
(A) Results of *AtGLP1* probed with a 386-bp DNA fragments of *AtGLP1*.
(B) Results of *AtGLP2* probed with a 386-bp DNA fragments of *AtGLP2*.

3.2 DNA gel blot hybridization

We studied the genomic organization of *AtGLP1* and *AtGLP2*. Both probes were hybridized to each of a single band of genomic DNA fragments digested with each one of four restriction endonucleases (**Fig. 2**). The results shown in **Fig. 2** are for low stringency washing. Probes used for these studies were 386-bp DNA fragments with 65% identity and GC% were 53% (*AtGLP1*), 49% (*AtGLP2*). Even by low stringency washing, ^{32}P-labelled probes could clearly distinguish the two closely related genes.

3.3 RNA gel blot hybridization

We examined the organ-specific accumulation of mRNAs for *AtGLP1* and *AtGLP2* by RNA gel blot hybridization (**Fig. 3**). *AtGLP1* mRNA and *AtGLP2* mRNA were detected in all overground organs but were not detected in roots. Leaves and flowers produced especially strong signals. As with *PnGLP* and *SaGLP*, the steady-state level of mRNAs for *AtGLP1* and *AtGLP2* fluctuated with the light/dark cycles. Results of leaves harvested at 4 h of light period ['Zeitgeber' time (zt)4] and at 8 h of darkness (zt16) are presented.

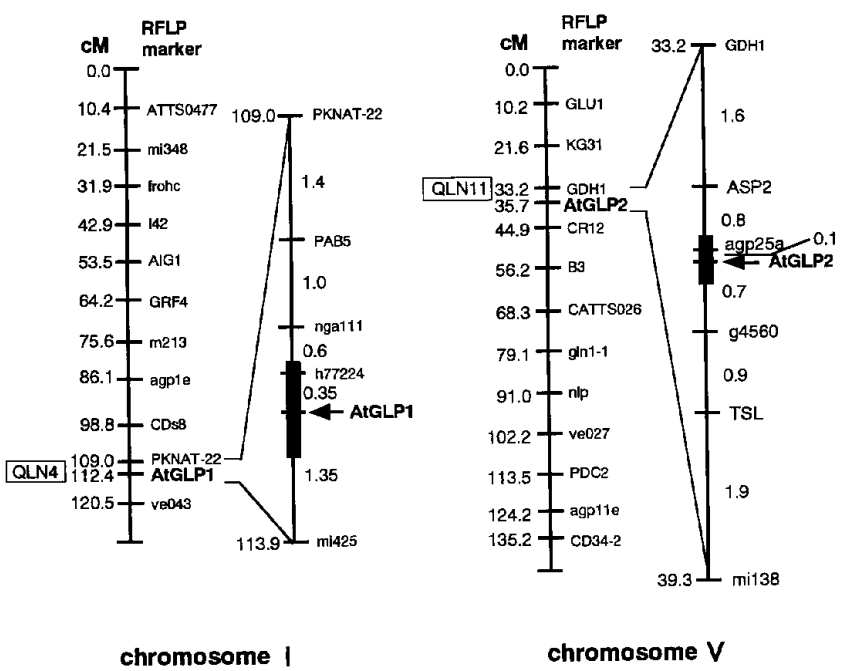

Fig. 4 Map position of *AtGLP1* and *AtGLP2* in *Arabidopsis* chromosomes. Linkage analysis was performed using the MAPMAKER (Macintosh version 2.0) mapping program of Lander *et al.* [16]. Linkage between loci was a minimum LOD score of 6.0. RFLP markers are designated by clone numbers. Distances between RFLP markers are given in centimorgans utilizing the Kosambi mapping function [25]. Chromosome number is indicated at the bottom of each map. QLN4 and QLN11 are QTLs detected in Landsberg *erecta* x Columbia RIL (recombinant inbred lines) population by Jansen *et al.* [17].

3. 4 RFLP mapping

RFLP mapping was performed to analyze the positional relationships between *AtGLP* genes and the mutants which had been characterized and mapped. We used the recombinant inbred (RI) lines (F8; $n=100$) derived from Columbia x Landsberg *erecta* [15] to determine the chromosomal location of *AtGLP1* and *AtGLP2*. *AtGLP1* was mapped on chromosome 1 at 112.4±0.6 cM. *AtGLP2* was mapped on chromosome 5 at 35.7±0.3 cM.

4. Discussion

Here, we described the molecular identification of homologs of *PnGLP* and *SaGLP* in *Arabidopsis thaliana*, namely, *AtGLP1* and *AtGLP2*. As we discussed previously, the molecular phylogenic tree clearly demonstrated that *PnGLP*, *SaGLP*, *AtGLP1* and *AtGLP2* constitute a distinct subfamily of GLPs. We previously named this group of protein as leaf-specific GLPs [7]. Although wheat germin was shown to be an oxalate oxidase (EC 4.3.2.1) [4], *PnGLP* and *SaGLP* reportedly might not have an oxalate oxidase activity [7, 8]. Currently, we have no direct evidence as to the function of leaf-specific GLPs. We previously speculated the physiological roles of leaf-specific GLPs in photoperiodism, including induction of flowering [7]. However, *Pharbitis* is not suitable for studying the physiological function of leaf-specific GLPs from several points. *Pharbitis* is a recalcitrant plant for making transgenic plants. There are no available genetic mutants in *Pharbitis*. *PnGLP* of *Pharbitis* constitute small gene families in the genome [Ono *et al.*, unpublished results]. Therefore, we decided to use *Arabidopsis* to study physiological roles of leaf-specific GLP.

DNA sequencing of the primary PCR products resulted in two sequences that showed high similarity to *PnGLP* and *SaGLP*. Results of database searching also suggested that there were only two leaf-specific GLPs in *Arabidopsis* genome, but at least 8 GLPs in the *Arabidopsis* ESTs. Therefore we concluded that we could determine both leaf-specific GLPs, namely, *AtGLP1* and *AtGLP2*. Moreover, the results of DNA gel blot analysis demonstrated that *AtGLP1* and *AtGLP2* were single copy genes. This enable us to perform molecular and genetical studies on the physiological function of leaf-specific GLPs.

We performed RNA gel blot hybridization to study the expression pattern of mRNA for *AtGLP1* and *AtGLP2*. As shown in **Fig. 3**, mRNA of *AtGLP1* and *AtGLP2* were detected in all overground organs of 3-month-old plants but were not detected in roots. These results were mostly consistent with the results of *SaGLP* [8], reflecting the close generic relationship between *Arabidopsis* and *Sinapis*. In *Pharbitis*, *PnGLP* was detected only in cotyledons and leaves [7]. Accumulation of mRNAs for *AtGLP1* and *AtGLP2* in leaves was regulated by light/dark cycles. These results are comparable to those for *PnGLP* and *SaGLP*. As GLPs other than leaf-specific GLPs reportedly did not show circadian regulation, this is a characteristic feature of leaf-specific GLPs, namely, *PnGLP*, *SaGLP*, *AtGLP1* and *AtGLP2*. However, the precise timing of the oscillation of mRNA level of GLPs during the photoperiod differed with the species. We are currently studying the timing of oscillation in detail.

AtGLP1 and *AtGLP2* were mapped on chromosome 1 and chromosome 5, respectively, and, did not correspond to any characterized mutants. As *AtGLP1* and *AtGLP2* are highly similar in structure as well as pattern of expression, they may complement each other. This might be why there are no reported mutants at these loci. However, some quantitative trait loci (QTL) of flowering time have been mapped around *AtGLP1* and *AtGLP2*. Jensen *et al.* reported QLN4 in the bottom region of chromosome 1 and QLN11 in the top region of chromosome 5 [17, 18]. *AtGLP1* and *AtGLP2* may correspond to these QTL. Moreover, a number of genes that are involved in floral development have been mapped to the top arm of chromosome 5 [19], *e.g. TOUSLED* (*TSL*) [20], *PISTILLATA* (*PI*) [21], *CONSTANS* (*CO*) [22] and *MALE STERILE 1* (*MS1*) [23]. *AtGLP2* may relate to some of these genes.

Membré *et al.* [24] quite recently reported the molecular cloning and characterization of cDNAs of *AtGER1* and *AtGER3* which are identical to *AtGLP1* and *AtGLP2*, respectively, at least in their nucleotide and amino-acid sequences. Our findings are similar to theirs, but, there are several differences. In the results of genomic DNA gel blot hybridization, Membré *et al.* showed that the size of the *Eco*RI fragment band that hybridized to the probe of *AtGER1* (*AtGLP1*) was much higher than 9.1 kbp. However, our results indicated that the *Eco*RI band containing *AtGLP1* (*AtGER1*) was 7.0 kbp (**Fig. 2**). Results of RFLP mapping also differed. Membré *et al.* reported that *AtGER1* was mapped on chromosome 5, whereas, we mapped *AtGLP1* (*AtGER1*) on chromosome 1. The results of RNA gel blot hybridization differed at several points. The most important difference was that we found significant fluctuation in the steady-state levels of mRNA in leaves with the light/dark cycles in *AtGLP1* (*AtGER1*), while they did not. Membré *et al.* also reported the absence of transcripts of *AtGLP2* (*AtGER3*) detected in green siliques while we obtained a positive band (**Fig. 3**). The discrepancy between the two sets of results may be due to

different conditions of cultivation. We are currently analyzing the effects of culture conditions on the transcription of *AtGLP1* and *AtGLP2* in detail.

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