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Characterization of the *GLP13* gene promoter in *Arabidopsis thaliana*

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Abstract

In transgenic plants, for many applications it is important that the inserted genes are expressed in a tissue-specific manner. This in turn could help better understanding their roles in plant development. Germin-like proteins (GLPs) play diverse roles in plant development and defense responses. In order to understand the functions and regulation of the *GLP13* gene, its promoter (762 bp) was cloned and fused with a β-glucuronidase (*GUS*) reporter gene for transient expression in *Arabidopsis thaliana* and tobacco (*Nicotiana tabacum* cv. K326). Histochemical analysis of the transgenic plants showed that *GUS* was specifically expressed in vascular bundles predominantly in phloem tissue of all organs in *Arabidopsis*. Further analyses in transgenic tobacco also identified similar *GUS* expression in the vascular bundles.

Additional key words: germin-like proteins, GUS activity, *Nicotiana tabacum*, phloem, tobacco, transgenic plants.

Introduction

Phloem plays important roles not only in material transport but also in signal transmission, mechanical support, and defence responses (Shah 2009, Turgeon *et al*. 2009). The long-distance transport between organs in phloem can be used by plant pathogens, such as viruses, viroids, phytoplasmas, and phloem bacteria for efficient propagation within the plant. Additionally, the high sugar and free amino acid content in phloem sap become the perfect diet for numerous insects (Douglas *et al*. 2006, Brzin *et al*. 2011). Recent studies indicate that the phloem response to pests and pathogens is probably a key element in plant defence (Shah 2009, Beneteau *et al*. 2010).

Based on the aforementioned evidence, phloem specific promoters could be important to direct the expression of disease-resistance genes in plants. For example, a *dENP* promoter of pumpkin displays a highly efficient and phloem-specific expression of a foreign gene and can be useful for the improvement of aphid-resistance or disease-resistance in developing transgenic potato plants (Guo *et al*. 2004).

Germin-like proteins (GLPs) are ubiquitous plant proteins encoded by diverse multigene families and have been identified in *Arabidopsis* (Membre *et al*. 1997, Carter *et al*. 1998), rice (Manosalva *et al*. 2009), soybean (Lu *et al*. 2010), sorghum (Paterson *et al*. 2009), barley (Zimmermann *et al*. 2006), wheat (Caliskan *et al*. 2004), and peanut (Chen *et al*. 2011). A number of reports have shown that GLPs are involved in plant defense responses (Manosalva *et al*. 2009, Breen *et al*. 2010, Lu *et al*. 2010). So far, only promoters of the barley *GER4c* gene were found to be expressed in response to pathogen attack (Himmelbach *et al*. 2010). We have shown that *GLP13* plays an important role in response to oxidative stress in *Arabidopsis* (Tang *et al*. 2011). In this report, we found that the expression of the *GLP13* promoter was active mainly in vascular bundles.

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Abbreviations: At - *Arabidopsis thaliana*; GLPs - germin-like proteins; GUS - β-glucuronidase.
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Materials and methods

Arabidopsis thaliana L. ecotype Columbia and *Nicotiana tabacum* L. cv. K326 plants were grown either in axenic culture on Murashige and Skoog (MS) medium or in soil in the greenhouse under 16-h photoperiod, irradiance of 120 μmol m^{-2} s^{-1}, temperature of 23 °C and relative humidity of 65 - 70 %. For *in vitro* experiments, seeds were surface-sterilized in 5 % sodium hypochlorite for 3 min and then in 0.15 % *Tween 20* for 3 min. The seeds were rinsed in distilled water and placed on MS plates (1× MS salts, 1.5 % sucrose and 0.8 % agar, pH 5.7). The plates were cold-treated at 4 °C for 3 d before grown vertically under continuous irradiance of 120 μmol m^{-2} s^{-1} as described previously (Lim *et al*. 2008).

To obtain the *GLP13* promoter, a 762 bp upstream of the start codon of the *GLP13* gene, which included the 5'-untranslated region of At3g10080 and part of the 3'-untranslated region of At3g10090, was amplified by PCR (30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s) using the primers F5′-GTCGACaatgtt atatcgtttgga-3′ and R5′- CCCGGGtttacttgaggtccatgtg-3′. The *Sal* I and *Smal* I sites are in capital letters and the sequences corresponding to the upstream region of *GLP13* are in small letters. The PCR products were digested and the *Sal* I-*Smal* I fragment was inserted into the corresponding restriction sites of p*BI101* to fuse the *GLP13* promoter to GUS.

The constructed binary vectors were introduced into *Agrobacterium tumefaciens* strain EHA105 and using the floral-dip method (Clough and Bent 1998, Studart *et al*. 2006), they were transformed into *Arabidopsis thaliana*. The transgenic seedlings were selected on MS agar plates with 50 mg dm^{-3} kanamycin. Following the *Agrobacterium*-mediated leaf disc transformation method (Horsch 1985), *pGLP13::GUS* construct was also transformed into tobacco and transgenic plants were selected according to resistance to 100 mg cm^{-3} kanamycin.

Transgenic lines containing the *pGLP13::GUS* reporter fusion construct were selected and analyzed for GUS expression. The organs (flowers, stems, cauline leaves, and rosette leaves) from 3 and 10-d-old seedlings were treated with GUS staining buffer (5 mM potassium hexocyanine ferrate II and III, 0.5 M EDTA, 1 M sodium phosphate buffer, 0.1 % *Triton X-100* and 20 mM X-Gluc, pH 7.0) at 37 °C in the dark for 3 to 5 h. For transgenic tobacco, samples were dipped in GUS reaction solution for 1 h, then vacuumed and incubated at 37 °C for 16 h. After that, the materials were stabilized with ethanol:acetic acid solution (3: 1, v/v) for 1 h, then decolorized and dehydrated progressively with 25 - 100 % ethanol. Finally the samples were imbedded in paraffin and cut into 8 μm paraffin sections which were deparaffined and observed under a *Stemi 2000-C* stereo microscope (*Carl Zeiss*, Jena, Germany).

Results and discussion

Using *PLACE/Signal Scan* (http://www.dna.affrc.go.jp/sigscan/signal1.pl) (Prestridge 1991, Higo *et al*. 1999), we found several *cis*-regulatory elements in the isolated *GLP13* promoter (Table 1). The *GLP13* 762-bp promoter

Table 1. Regulatory elements found in promoter of *GLP13*.

Regulatory element	No.	Location	Functions	Reference
CAAT	7	-32,-88,-97,-187,-199,-287, -494,	tissue specific promoter activity	Shirsat *et al*. 1989
GTAC (copper-response element)	6	-14,-449,-454,-507,-523, -570	involved in oxygen-response of Cyc6 and Cpx1 genes	Quinn *et al*. 2002
ATATT	5	-323,-368,-429,-588,-754	root specific expression	Elmayan *et al*. 1995
CCAAT	4	-87,-96,-186,-198	heat shock protein activity	Wenkel *et al*. 2006
AAAG	4	-118,-341,-537,-579,	binding site for D of proteins	Yanagisawa *et al*. 1999
CT/GA	3	-83,-215,-573	vascular specific expression	Roberto *et al*. 2011
GATA	3	-265,-273,-401,	light regulated and tissue specific gene expression	Lam *et al*. 1989
TGAC(Y) (core sequence in W box)	3	-84,-216,-327	pathogen inducibility, gibberellin signaling pathway	Eulgem *et al*. 2000, 2007
GAAAAA	2	-101,-488	pathogen and NaCl induced	Park *et al*. 2004
TATTAAT	2	-706,-668	transcription initiation pathogen inducibility	Eulgem *et al*. 2000
TAAAG	2	-117,-578	guard cell-specific gene expression	Plesch *et al*. 2001
AGAAA	2	-581,-7000	pollen specific expression	Filichkin *et al*. 2004

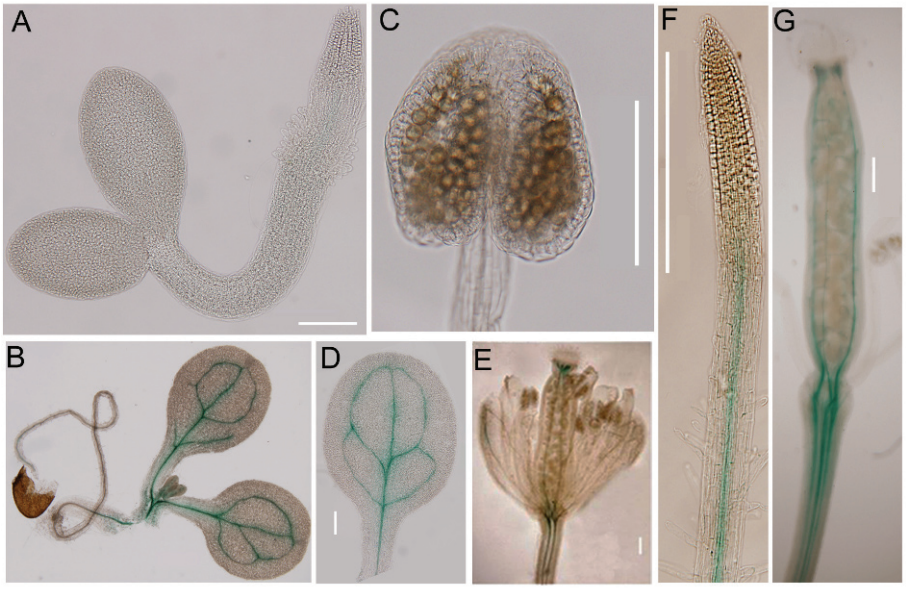

Fig. 1. The histochemical localization of GUS activity in transgenic plants of *Arabidopsis* carrying a 762 bp *pGLP13::GUS* construct. GUS activity is visualized in blue in 1-d-old seedling (*A*), 3-d-old seedling (*B*), anther (*C*), rosette leaf (*D*), flower (*E*), primary root tip (*F*), and silique (*G*). The *scale bar* was 200 µm.

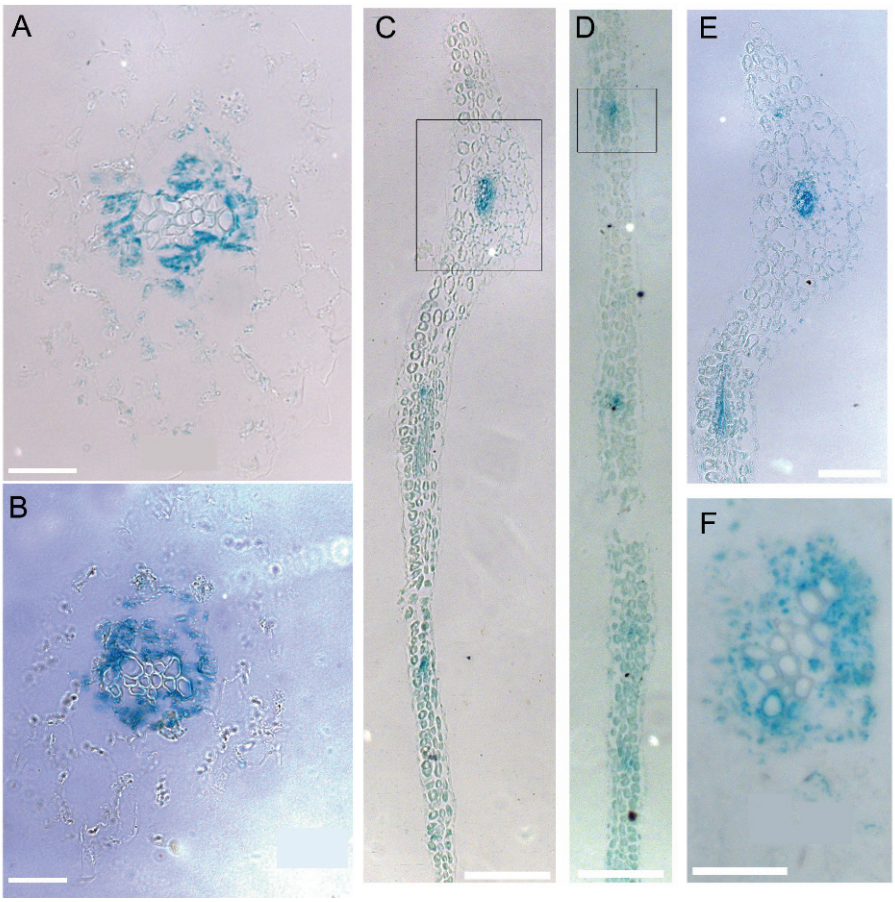

Fig. 2. Paraffin section of GUS-expressing plants of *Arabidopsis*. GUS activity is visualized in blue. Transverse section of root (*A*), stem (*B*) and leaf (*C,E*), and longitudinal section of leaf (*D,F*). The *scale bar* was 50 µm.

also have three putative W-boxes with a typical TGAC core motif which were reported to be involved in pathogen and elicitor induced responses (Eulgem *et al*. 2007, Himmelbach *et al*. 2010); three CT/GA motifs which potentially regulate expression within the vascular system (Ruiz-Medrano *et al*. 2011); two GAAAAA motifs which are part of the GT1 box for pathogen and NaCl-induced in SCaM-4 promoter (Park *et al*. 2004, Kovalchuk *et al*. 2010); and one TAAAG motif which was involved in guard cell-specific expression.

Histochemical staining revealed that a strong GUS activity was mainly in vascular bundle tissues of the transgenic *Arabidopsis* plants (Fig. 1). GUS activity was also observed in the cotyledon (Fig. 1*B*), primary root (Fig. 1*F*), silique (Fig. 1 *G*), rosette leaf (Fig. 1*D*), and stem (Fig. 1*H*). Slight GUS expression was observed

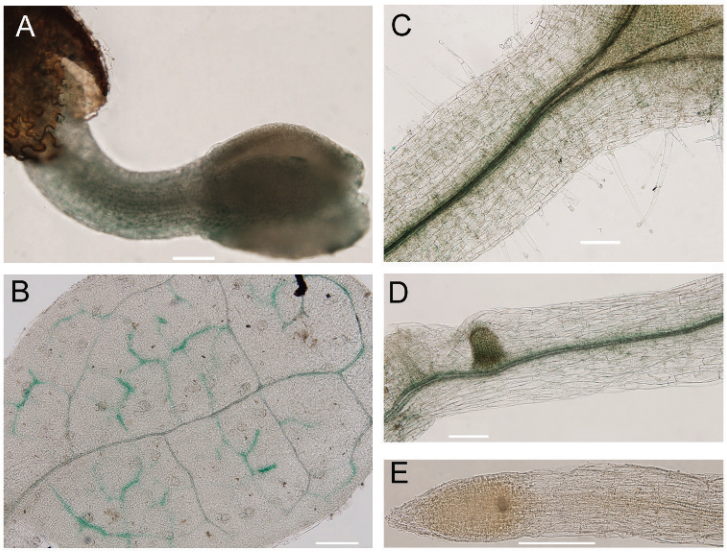

Fig. 3. Histochemical assay of GUS reporter gene in transgenic plants of tobacco. GUS activity is visualized in blue in 2-d-old seedling (*A*), leaf (*B*), petiole (*C*), lateral root (*D*), and primary root (*E*). The *scale bar* was 200 μm.

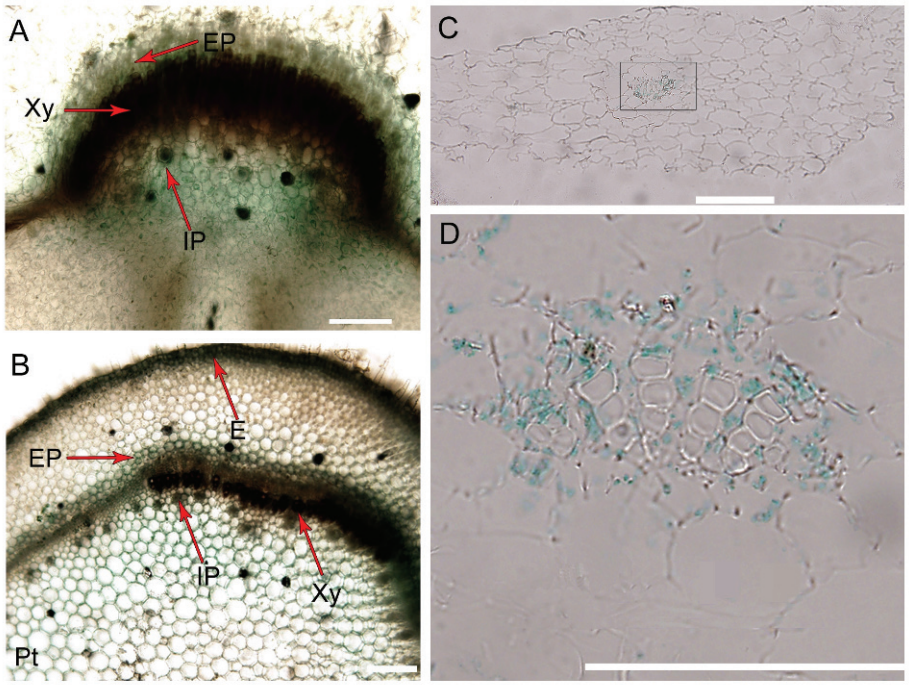

Fig. 4. Paraffin section and free-hand sections showing GUS reporter gene in transgenic plants of tobacco. GUS activity is visualized in blue in transverse section of stem in different times *(A,B)* and transverse section of leaf (*C,D*) [internal phloem (IP), epidermis (E), epiphloem (EP), pith (Pt), and xylem (Xy)]. The *scale bar* was 200 μm.

in the vascular bundles of 1-d-old seedling (Fig. 1*A*) and in flower (Fig. 1*E*). However, no expression was detected in the anther (Fig. 1*C*), seed coat, calyx, and sepal. The paraffin section's data also indicated that *GLP13* promoter was active in the vascular tissues of different organs (Fig. 2). Taken together, our results suggested that the *GLP13* gene promoter may be a vascular bundle-specific promoter.

In order to determine the tissue specificity of *GLP13* promoter, fifteen independent transgenic tobacco lines were examined for the presence of *GUS* gene. All positive lines showed the same pattern of GUS expression which was essentially the same as in transgenic *Arabidopsis* (Fig. 1). The strong GUS expression was observed mainly in vascular tissues, especially in leaf (Fig. 3*B*), petiole (Fig. 3*C*), and root (Fig. 3*D*), but no expression in root tips (Fig. 3*E*). In mature roots, GUS expression was detected in two vascular strands of the central cylinder (Fig. 3*D*). Normally, cells between two protoxylem strands differentiate into metaxylem generating a continuous file of xylem elements (Matsuda *et al*. 2002). Therefore, the staining pattern indicated that the expression of *pGLP13::GUS* was specific to phloem in tobacco as in *Arabidopsis*.

To determine more precisely the cell types in which the GLP13 promoter is active, 3 to 4 week old transgenic tobaccos were used for free-hand and paraffin sections (Fig. 4). In the stem, GUS expression was detected mainly in both internal and external phloem, slight expression in epidermis, but not in any other tissues such as cortex, pith, and xylem (Fig. 4*A,B*). In transverse sections of leaves, GUS expression was only in the phloem (Fig. 4 *C,D*).

The phloem transports nutrients, defensive compounds, and different signals. It constitutes a strategic checkpoint for defense against pathogens and pests (Van Bel *et al*. 2004, Turgeon *et al*. 2009). Significant effort is currently directed towards the characterization of phloem specific promoters and the expression of defense components (Himmelbach *et al*. 2010, Kovalchuk *et al*. 2010, Dutt *et al*. 2012). GLPs have been found in plants and their role in eliciting plant defense responses has been established (Dumas *et al*. 1995, Zhang *et al*. 1995, Wei *et al*. 1998, Lou *et al*. 2006, Zimmermann *et al*. 2006, Godfrey *et al*. 2007, Manosalva *et al*. 2009, Himmelbach *et al*. 2010, Chen *et al*. 2011). However, the characterization of their corresponding promoter regions has received little attention. Here we describe the isolation of the *GLP13* promoter fragment from *Arabidopsis* and its characterization in transgenic *Arabidopsis* and tobacco plants by constructing the *pGLP13::GUS* fusion. Through histochemical detection of GUS expression, we found GUS expression mainly evident in vascular bundle tissues in both *Arabidopsis* (Fig. 1) and tobacco (Fig. 3). GUS expression was detected in 2-d-old seedling (Fig. 3*A*) and lateral root (Fig. 3*E*) only in vascular bundle tissue. GUS expression in transgenic plants were further analyzed on free-hand and paraffin sections. We confirmed that *GLP13* was expressed predominantly in the phloem of all organs and there was no conspicuous difference between *Arabidopsis* (Fig. 2) and tobacco (Fig. 4). Thus constitutive vascular expression of the *GLP13* promoter may be sufficient to direct expression of the corresponding products in response to pathogen attack. In addition, some consensus sequences motifs of organ-specific elements were identified through bioinformatics analysis of the *GLP13* promoter. W-boxes with a typical TGAC core motif (Eulgem *et al*. 2007, Himmelbach *et al*. 2010) and GT1 box with GAAAA motif (Park *et al*. 2004, Kovalchuk *et al*. 2010) were detected and many of them were reported to be involved in pathogen and elicitor induced responses. CT/GA motifs were found in *GLP13* promoter which regulate expression within the vascular system (Ruiz-Medrano *et al*. 2011). Based on the analysis of the *GLP13* promoter, we can conclude that *GLP13* promoter can be used to target defense proteins against pathogens that reside in phloem.

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