Running title: GATEWAY BINARY VECTORS FOR EFFICIENT CLONING

Title: Development of Series of Gateway Binary Vectors, pGWBs, for Realizing Efficient Construction of Fusion Genes for Plant Transformation

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ABSTRACT

We developed a new series of binary vectors useful for Gateway cloning to facilitate transgenic experiments in plant biotechnology. The new system, Gateway Binary Vectors (pGWBs) realized efficient cloning, constitutive expression using the cauliflower mosaic virus (CaMV) 35S promoter and the construction of fusion genes by simple clonase reaction with an entry clone. The reporters employable in this system are GUS, sGFP, LUC, EYFP, and ECFP. The tags available are 6xHis, FLAG, 3xHA, 4xMyc, 10xMyc, GST, T7-epitope, and tandem affinity purification (TAP). In total, 13 kinds of reporter or tag were arranged and were almost applicable to both N- and C-fusions. The pGWBs could be used for many purposes, such as promoter::reporter analysis, observation of subcellular localization by the expression of proteins fused to a reporter or tag, and analysis of protein-protein interaction by copurification and immunodetection experiments. The pGWBs were constructed with modified pBI101 containing a CaMV35S promoter-driven hygromycin phosphotransferase (*HPT*) gene as the second selection marker. We also constructed pGWBs with the marker *HPT* driven by the nopaline synthase promoter. By using the pGWB system, the expression of tagged proteins, and the localization of GFP-fused proteins were easily analyzed. Moreover, tissue-specific and inducible gene expression using a promoter was also monitored with pGWBs. It is expected that, the pGWB system will serve as a powerful tool for plasmid construction in plant research.

[Key words: binary vector, gateway cloning, plant, reporter, tag, transformation]

Introduction

Plant genome projects have provided much sequence information and, as a result, genome wide analyses of plants are now underway on a global scale (1, 2). In these studies, systematic analysis of the basic characteristics of genes and their products, that is, patterns of expression and subcellular localization, is very important and gene fusion to a reporter or tag is a convenient and powerful method of functional analysis *in planta*. These techniques are also effective in the detailed analysis of gene networks including colocalization, complex formation and interactions of gene products. For such analysis, the introduction of a fusion gene is necessary and, in most cases, is carried out by *Agrobacterium*-mediated transformation using a Ti binary vector. However, in this transformation system, conventional methods for cloning of the transgene in the binary vector with restriction enzymes and ligase are time consuming and often laborious because binary vectors are very large and have many restriction enzyme recognition sites. This technical barrier makes it difficult to carry out functional analysis using fusion genes to a reporter or tag *in vivo*. To overcome this, a new cloning system that allows the efficient and reliable construction of fusion genes for plant research is therefore necessary.

Gateway cloning employs reversible recombination between *att*B, P, L, and R sites, enabling directional cloning into a plasmid using one simple reaction and is particularly advantageous in the construction of fusion genes (3). In this report, we describe the construction of gateway-compatible binary vectors, namely, Gateway Binary Vectors (pGWBs), that have the capability to fuse with many kinds of reporter and epitope tag. Fusion to fluorescent proteins is always applied to the analysis of subcellular localization, colocalization and fluorescent resonance energy transfer (FRET). In the pGWB system, β-glucuronidase (GUS) (4), luciferase (LUC) (5), synthetic green fluorescent protein with S65T mutation (sGFP) (6, 7), enhanced yellow fluorescent protein (EYFP) (8), and enhanced cyan fluorescent protein (ECFP) (8) can

be used as well as in other gateway-compatible systems (9-11). Epitope tagging is also useful for analyzing protein-protein interactions by coprecipitation and immunodetection. The pGWB system also employs epitopes including repeat type, hexa histidine (6xHis), FLAG (12), triple HA (3xHA) (13), 4 repeats of Myc (4xMyc) (13), 10 repeats of Myc (10xMyc) (13), glutathione S-transferase (GST) (14), T7-epitope (15), and tandem affinity purification (TAP) (16) for the one-step construction of tagging. Promoter::reporter constructs are routinely used to reveal the temporal and spatial regulations of gene expression. Here, we tested the use of the pGWB system in promoter analysis, showing agreement with the result obtained using conventional binary vectors.

MATERIALS AND METHODS

Plasmid construction Standard methods of plasmid construction were used (17). In general, gateway-compatible vectors were firstly constructed on pUC119 (pUGW series) and then transferred to binary vectors (pGWB series). The regions generated by PCR, the direct cloning of oligo DNA, and the ligated junctions were confirmed by sequencing in all vectors. In the PCR, KOD DNA polymerase (Toyobo, Tokyo) was used to generate amplified products with blunt ends. All linkers, adapters and primers are listed in Table 1.

TABLE 1

pUGW1, pUGW3, pUGW4, pUGW5, and pUGW6 were constructed differently from the other pUGWs as follows. For pUGW1, the *Xba*I-*Eco*RI fragment of pBI101 containing GUS and nopaline synthase terminator (NosT) was cloned into the same site of pUC119 and designated pBI401. The *Sac*I-*Xba*I-*Sac*I adapter was introduced into the *Sac*I site located between GUS and NosT of pBI401 then the GUS fragment of the resulting plasmid was replaced with Gateway reading frame cassette A (Invitrogen, Carlsbad, CA, USA) using *Xba*I followed by treatment with the klenow enzyme. Finally, the *Xho*I linker was introduced into the blunted *Not*I site located downstream

of *att*R1 in the resulting vector to construct pUGW1. The introduction of the *Xho*I linker into this site was carried out as the final step for the construction of pUGW1, pUGW3, pUGW4, pUGW5, and pUGW6 described below. For pUGW3, pBI401 was digested by *Xba*I and treated with the klenow enzyme then Gateway reading frame cassette C.1 (Invitrogen) was introduced. For pUGW4, the *Sac*I linker was introduced into the blunted *Not*I site of pTH2 (6). The resulting plasmid was designated pTH2S and Gateway reading frame cassette A was introduced into the blunted *Xba*I/*Nco*I site. For pUGW5, the cauliflower mosaic virus (CaMV) 35S promoter of pTH2S was replaced with the CaMV35S promoter of pBI121 using *Hin*dIII and *Xba*I. The resulting plasmid was designated pTH121 and Gateway reading frame cassette C.1 was introduced into the blunted *Xba*I site. For pUGW6, Gateway reading frame cassette A was introduced into the blunted *Bsr*G1 site of pTH121.

For the construction of the remaining pUGWs, the platform vectors pUGW2 and pUGW0 were firstly made. The *Hin*dIII-*Eco*RI fragment of pBI121 containing CaMV35S::GUS-NosT was cloned into the same site of pUC119 and designated pBI421. The GUS fragment of pBI421 was replaced with the *Xba*I-*Hpa*I-*Aor*51HI-*Sac*I adapter using *Xba*I and *Sac*I then Gateway reading frame cassette B (Invitrogen) was introduced into the *Hpa*I site. Next, the *Hin*dIII-*Xba*I-*Hin*dIII adapter was introduced into the *Hin*dIII site located upstream of the CaMV35S promoter in the resulting plasmid. Finally, the *Xho*I linker was introduced into the blunted *Not*I site located just downstream of *att*R1 and designated pUGW2. The GUS fragment of pBI421 was replaced with *Xba*I-*Aor*51HI-*Hpa*I-*Sac*I using *Xba*I and *Sac*I then Gateway reading frame cassette A was introduced into the *Hpa*I site. Finally, the *Xho*I linker was introduced into the blunted *Not*I site of the resulting plasmid and designated pUGW0.

The pUGWs for fusion to the C-terminal (CaMV35S, C-fusion) were made from pUGW2 by introducing a reporter or tag sequence into the *Aor*51HI site. Oligo

DNAs of 6xHis, FLAG, and T7-epitope were used for the construction of pUGW8, pUGW11, and pUGW26, respectively. The 3xHA sequence was prepared by PCR using pFA6a-3HA-kanMX6 (13) as a template with 3xHA primers. After PCR, the amplified product was digested by *Sma*I and *Aor*51HI and used for the construction of pUGW14. The 4xMyc and 10xMyc sequences were prepared by PCR using pFA6a-13Myc-KanMX6 (13) as a template with 4-10xMyc primers. Because the same DNA sequence was used for the 1st, 4th, 7th, 10th, and 13th Myc in the template, a repeat ladder appeared after PCR. Amplified products corresponding to 4xMyc and 10xMyc were prepared after separation by agarose gel electrophoresis, digested by *Sma*I and *Aor*51HI and used for the construction of pUGW17 and pUGW20, respectively. The GST sequence was prepared by PCR using pGEX2T (Amersham-Pharmacia, Piscataway, NJ, USA) as a template with GST primers. The amplified product was used for the construction of pUGW23. The TAP sequence was prepared by PCR using pFA6a-TAP-kanMX6 as a template (16) with TAP primers. The amplified product was used for the construction of pUGW29. The LUC sequence was prepared by PCR using pGV-B2 (Toyo B-Net, Tokyo) as a template with LUC primers. The amplified product was used for the construction of pUGW36. The EYFP sequence was prepared by PCR using pEYFP (Clontech, Mountain View, CA, USA) as a template with EYFP primers. The amplified product was used for the construction of pUGW41. The ECFP sequence was prepared by PCR using pECFP (Clontech) as a template with same primers for EYFP and the amplified product was used for the construction of pUGW44. For the construction of a no-promoter version of the C-fusion vector, the CaMV35S promoter was removed by *Xba*I from pUGW8, pUGW11, pUGW14, pUGW17, pUGW20, pUGW23, pUGW26, pUGW36, pUGW41, and pUGW44 to construct pUGW7, pUGW10, pUGW13, pUGW16, pUGW19, pUGW22, pUGW25, pUGW35, pUGW40, and pUGW43, respectively (no promoter, C-fusion).

The pUGWs for fusion to the N-terminal (CaMV35S, N-fusion) were made from pUGW0 by introducing a reporter or tag sequence into the *Aor*51HI site, as described

for the C-fusion vectors. pUGW9, pUGW12, pUGW15, pUGW18, pUGW21, pUGW24, pUGW27, pUGW42, and pUGW45 were employed as vectors for the N-terminal fusions of 6xHis, FLAG, 3xHA, 4xMyc, 10xMyc, GST, T7, EYFP, and ECFP, respectively. The pUGWs were selected on media containing 50 μg/ml ampicillin and 30 μg/ml chloramphenicol.

The *Hin*dIII-*Sac*I or *Xba*I-*Sac*I fragments of the pUGWs were introduced into the same sites of pABH-Hm1 (18) to construct corresponding pGWBs (pGWB1–45). Because the TAP sequence included a *Sac*I site, the *Sac*I-*Sac*I fragment containing part of the TAP sequence was restored for the construction of pGWB28 and pGWB29. pGWBs containing the nopaline synthase promoter (NosP)::hygromycin phosphotransferase (HPT)-NosT as a hygromycin-resistant marker were constructed as follows. The *Eco*RI fragment of pYLTAC7 (19) containing NosP::HPT-NosT was cloned into the *Eco*RI site of pBI121 and the resulting vector was designated pBI121-NosHyg. The *Hin*dIII-*Sac*I or *Xba*I-*Sac*I fragments of pUGW3, pUGW4, pUGW28, and pUGW35 were introduced into the same site of pBI121-NosHyg to construct pGWB203, pGWB204, pGWB228, and pGWB235, respectively. The pGWBs were selected on media containing 50 μg/ml kanamycin, 50 μg/ml hygromycin and 30 μg/ml chloramphenicol.

Construction of entry clones and introduction into pGWBs The *att*B1 and *att*B2 sequences were added to the N- and C-terminals of the GUS coding sequence, respectively, by two-step adapter PCR as described by the manufacturer (Invitrogen). The template was pBI121 and GUS-*att*B1 (forward) and GUS-*att*B2 (reverse) were used as primers for the 1st PCR. The 2nd PCR was performed with the *att*B1 and *att*B2 primers, then the amplified product was introduced into pDONR201 by BP reaction as described by the manufacturer (Invitrogen). The *ATSEC13* (*Arabidopsis thaliana SEC13*, AT2g30050) coding sequence was amplified from genomic DNA and cloned into pDONR201 as described above. *ATSEC13*-*att*B1 (forward) and

*ATSEC13-att*B2 were used for 1st adapter PCR. A 3888-bp upstream fragment of *PHT1* (20) was amplified from genomic DNA by PCR with *PHT1* promoter primers, then cloned into pENTR D-TOPO, as described by the manufacturer (Invitrogen). The primers are listed in Table 1. The transfer of the DNA fragment from the entry clone to pGWB by LR reaction was performed as described by the manufacturer (Invitrogen). Recombinant pGWBs were selected on media containing 50 μg/ml kanamycin and 50 μg/ml hygromycin.

Plant materials and transformation The culture and stable transformation of *Nicotiana tabacum* cv. BY-2 cells were carried out as described by Matsuoka and Nakamura (21). *A. thaliana* ecotype Columbia was grown at 22 ºC under a 16L/8D light condition and transformed using a floral dip procedure (22).

Fluorescence microscopy BY-2 cells were viewed using a fluorescence microscope (BX51; Olympus, Tokyo) equipped with U-MGFPHQ and U-MYFPHQ fluorescence mirror units (Olympus) for GFP and yellow fluorescent protein (YFP) detection, respectively.

Histochemical and quantitative GUS activity assays Inorganic phosphate (Pi) treatment, histochemical GUS staining and quantitative GUS assay of transgenic *A. thaliana* were performed as described by Koyama *et al*. (20). A suspension culture of transgenic BY-2 cells was spun briefly and stained for GUS as described above.

Immunoblot analysis Protein preparation from transgenic BY-2 cells and immunoblot analysis were performed as described previously (23). Anti-GUS polyclonal antibody (24), anti-GFP polyclonal antibody (Clontech), anti-FLAG M2 monoclonal antibody (Sigma-Aldrich, St.Louis, MO, USA), anti-HA monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-Myc

monoclonal antibody 9E10 (Santa Cruz Biotechnology) were used. Dr. Western (Oriental Yeast, Tokyo) was used as a marker. Chemical luminescence was captured by Light Capture (ATTO, Tokyo).

Nucleotide accession number of pGWBs The complete nucleotide sequence of pGWBs reported in this paper has been submitted to the DDBJ nucleotide sequence database under accession nos. AB289764 to AB289803.

RESULTS AND DISCUSSION

Structure of pGWBs We constructed a number of binary vectors useful in Gateway cloning. The backbone of our pGWBs was a modified pBI containing *HPT* and the neomycin phosphotransferase II (*NPTII*) gene; thus, all pGWBs conferred both kanamycin and hygromycin resistance. We used two types of hygromycin marker, CaMV35S::HPT-NosT and NosP::HPT-NosT. pGWB vectors incorporating NosP::HPT-NosT (pGWB200s) were constructed to avoid the possible enhancer effect of CaMV35S in promoter analysis. The orientation of NosP::HPT-Tnos was the same as that of the gene cloned by the LR reaction.

The structure of the T-DNA region of pGWBs containing the 35S::HPT-NosT marker is illustrated in Fig. 1A. pGWB1 was used for simple cloning, pGWB2 for overexpression using the CaMV35S promoter, and pGWB3 for promoter::GUS analysis. pGWB4-27 and 40-45 were used for various fusions. Three types of fusion, namely, no promoter and C-fusion, CaMV35S promoter and C-fusion, and CaMV35S promoter and N-fusions, were performed with 10 kinds of reporter and tag (sGFP, 6xHis, FLAG, 3xHA, 4xMyc, 10xMyc, GST, T7, EYFP, and ECFP). pGWB28 was used for TAP analysis with its own promoter and pGWB29 for TAP analysis with the CaMV35S promoter. pGWB35 was used for promoter::LUC analysis. The structure of the T-DNA region of pGWB200s is illustrated in Fig. 1B. pGWB203, pGWB204, and pGWB235 were used for promoter::reporter analysis with

FIG. 1

a NosP-driven HPT marker. pGWB228 was used in the promoter::protein-TAP construct with a NosP-driven HPT marker.

All pGWBs have a unique *Xho*I site just downstream of *att*R1 for linearization and to facilitate the LR reaction. The frame of all pGWBs was adjusted to the general rule in Gateway cloning. Figure 2 shows the linker sequences generated after the LR reaction. The peptide sequence derived from *att*B2 becomes the linker for C-fusion and that of *att*B1 becomes the linker for N-fusion. These sequences did not disturb enzyme activity or the localization of the protein as described below.

FIG. 2

Expression of GUS fusion protein in stably transformed BY-2 cells To investigate protein fusion by pGWBs *in vivo*, the GUS coding sequence was transferred from the entry clone to pGWB2, pGWB6, pGWB8, pGWB11, pGWB12, pGWB15, pGWB18, and pGWB20, designated pGWBs-*GUS*, then introduced into BY-2 cells. Transformed calli appearing on the selection plate were firstly examined by GUS staining. GUS-positive calli were selected and cultured in liquid media for further analysis. GFP fluorescence was detected in the BY-2 cells transformed with pGWB6-*GUS*, indicating the expression of the active sGFP-GUS fusion protein (Fig. 3A). Next, the expression of GUS fusion proteins was analyzed by immunoblotting. In the blot with anti-GUS antibody, GUS proteins were detected in various sizes depending on the reporter or tag fused (Fig. 3B). For the pGWB18-*GUS* and pGWB20-*GUS* constructs, two bands were detected, a small band with the same size as a native GUS protein and a large band corresponding to Myc-tagged GUS. Fusion accuracy was examined by immunoblotting with anti-GFP, anti-FLAG, anti-HA, and anti-Myc antibodies. The fusion proteins were detected with the corresponding anti-tag antibody at the same position as with the anti-GUS antibody (Fig. 3C-F), indicating that the tags were correctly fused to GUS. As described above, two bands were detected in Myc fusion with the anti-GUS antibody; however, only the large band was detected with the anti-Myc antibody (Fig. 3F), indicating that the Myc repeat was

FIG. 3

partly lost from the GUS fusion proteins under our experimental conditions. Overall, these results indicate that the expression of fusion proteins was correctly achieved using the pGWB system.

Analysis of subcellular localization using GFP fusion GFP fusion is a powerful technique for the analysis of the subcellular localization of proteins. To investigate the applicability of pGWBs in localization analysis, pGWB5 and pGWB6 were used to perform fusion of the ATSEC13 (*A. thaliana* SEC13) protein with sGFP. These constructs were designated pGWB5-*ATSEC13* and pGWB6-*ATSEC13*, respectively. SEC13 is a component of the COPII (coat protein II) necessary for the budding of vesicles from the ER (25), and the ATSEC13-GFP fusion protein constructed by conventional methods was observed to localize in the cytosol and at protein-export sites of the ER that showed a fluorescent pattern of perinuclear ring and intracellular dots in plant cells (26). BY-2 was transformed with pGWB5-*ATSEC13* or pGWB6-*ATSEC13* then intracellular GFP fluorescence was observed. The *A. thaliana* cytochrome b5 (Cyt b5) was used as a control of the ER localization (27). All three constructs showed the perinuclear ring distribution of GFP or YFP (Fig. 4), indicating that the GFP fusion protein constructed using the pGWB system localizes correctly in the cells.

FIG. 4

Analysis of gene expression using promoter::GUS fusion construct Promoter analysis using a reporter gene is commonly used to determine the expression pattern of a target gene. In addition, the isolation of promoter sequences that drive tissue-specific and inducible expression of target genes is very important in plant biotechnology. To examine the applicability of the pGWB system in promoter::GUS analysis, we chose the *PHT1* promoter, which encodes the *A. thaliana* phosphate transporter. *PHT1* is known to be root-specific and is induced by Pi starvation (20). Because the enhancer sequence of the CaMV35S promoter is thought to affect the

expression of neighboring genes, we constructed a *PHT1* promoter (3888 bp)::GUS fusion gene with pGWB3, which contains a CaMV35S-driven HPT marker, and pGWB203, which contains a NosP-driven HPT marker. These constructs were designated pGWB3-*PHT1* and pGWB203-*PHT1*, respectively, and introduced into *A. thaliana* then analyzed for GUS expression.

In histochemical analysis, both constructs showed strong GUS staining in the roots and only faint expression in above-ground organs under the low-Pi condition (Fig. 5A , B). Within the roots, no expression was observed in the root meristematic region (Fig. 5C, D), consistent with a previous study using a conventional binary vector system (20). Next, we analyzed GUS expression in response to Pi concentration by quantitative GUS assay with 4-methylumbelliferyl (MU)-β-D-glucuronide as substrate (20). The GUS-specific activities in the roots of pGWB3-*PHT1* and pGWB203-*PHT1* transgenic plants grown under a Pi-containing condition (200 μM) were 185 (mean n=23) and 33.8 nmol MU/min/mg protein (mean n=25), respectively. These activities increased to 328 (mean n=23) and 62.4 (mean n=25), respectively, after the transgenic plants were transferred to a Pi-starved condition (0 μM). The GUS-specific activities increased by 1.8-fold in both transgenic lines by phosphate deprivation. The GUS-specific activity in the roots was fivefold higher with pGWB3-*PHT1* than with pGWB203-*PHT1*. The CaMV35S promoter used to drive *HPT* may affect the expression level of the reporter *GUS*. The GUS-specific activities in the leaf of pGWB3-*PHT1* and pGWB203-*PHT1* transgenic plants under a Pi-containing condition were 14.7 (mean n=23) and 0.570 (mean n=25), respectively. These activities slightly increased to 20.6 (mean n=23) and 0.62 (n=25), respectively. Although the mRNA level of *PHT1* was not quantitatively analyzed in our experiment, the results of the quantitative GUS analysis were in accordance with the results of Northern blot analysis reported by other researchers (28). Our results showed that gene regulation by the *PHT1* promoter could be monitored using the pGWB system.

FIG. 5

In conclusion, the pGWBs constructed in this study are very useful for efficient

cloning in transgenic research, and particularly powerful for the construction of fusion genes. Once the entry clone is made, fusion constructs with many kinds of reporter or tag can be obtained using the one-step LR reaction. Our pGWB system has some distinct characteristics compared with other gateway-compatible binary vectors (9-11). Because all tags and reporters are equipped on the same backbone vector as for pBI-H1 (29) that is widely used in many transgenic researches, experiments are performed under uniform conditions. Both kanamycin and hygromycin were available for selection in plants. Also, a repeat epitope will enhance the sensitivity of detection. By using the pGWB system, expression, localization, functional and interaction analyses in plants could be accelerated.

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REFERENCES

1. **Arabidopsis Genome Initiative:** Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature, **408,** 796-815 (2000).
2. **International Rice Genome Sequencing Project:** The map-based sequence of the rice genome. Nature, **436,** 793-800 (2005).
3. **Walhout, A.J., Temple, G.F., Brasch, M.A., Hartley, J.L., Lorson, M.A., van den Heuvel, S., and Vidal, M.:** GATEWAY recombinational cloning: application to the cloning of large numbers of open reading frames or ORFeomes. Methods Enzymol., **328,** 575-592 (2000).
4. **Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W.:** GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J., **6,** 3901-3907 (1987).
5. **Sherf, B.A. and Wood, K.V.:** Firefly luciferase engineered for improved genetic reporting. Promega Notes, **49,** 14-21 (1994).
6. **Chiu, W., Niwa, Y., Zeng, W., Hirano, T., Kobayashi, H., and Sheen, J.:** Engineered GFP as a vital reporter in plants. Curr. Biol., **6,** 325-330 (1996).
7. **Niwa, Y.:** A synthetic green fluorescent protein gene for plant biotechnology. Plant Biotechnol., **20,** 1-11 (2003).
8. **Huang, J., Taylor, J.P., Chen, J.G., Uhrig, J.F., Schnell, D.J., Nakagawa, T., Korth, K.L., and Jones, A.M.:** The plastid protein THYLAKOID FORMATION1 and the plasma membrane G-protein GPA1 interact in a novel sugar-signaling mechanism in *Arabidopsis*. Plant Cell, **18,** 1226-1238 (2006).
9. **Karimi, M., Inze, D., and Depicker, A.:** GATEWAY vectors for *Agrobacterium*-mediated plant transformation. Trends Plant Sci., **7,** 193-195 (2002).
10. **Curtis, M.D. and Grossniklaus, U.:** A gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiol., **133,** 462-469

(2003).

11. **Earley, K.W., Haag, J.R., Pontes, O., Opper, K., Juehne, T., Song, K., and Pikaard, C.S.:** Gateway-compatible vectors for plant functional genomics and proteomics. Plant J., **45,** 616-629 (2006).

12. **Chubet, R.G. and Brizzard, B.L.:** Vectors for expression and secretion of FLAG epitope-tagged proteins in mammalian cells. Biotechniques, **20,** 136-141 (1996).

13. **Bahler, J., Wu, J.Q., Longtine, M.S., Shah, N.G., McKenzie, A., 3rd, Steever, A.B., Wach, A., Philippsen, P., and Pringle, J.R.:** Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. Yeast, **14,** 943-951 (1998).

14. **Smith, D.B. and Johnson, K.S.:** Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione *S*-transferase. Gene, **67,** 31-40 (1988).

15. **Yano, D., Sato, M., Saito, C., Sato, M.H., Morita, M.T., and Tasaka, M.:** A SNARE complex containing SGR3/AtVAM3 and ZIG/VTI11 in gravity-sensing cells is important for *Arabidopsis* shoot gravitropism. Proc. Natl. Acad. Sci. USA, **100,** 8589-8594 (2003).

16. **Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Seraphin, B.:** A generic protein purification method for protein complex characterization and proteome exploration. Nat. Biotechnol., **17,** 1030-1032 (1999).

17. **Sambrook, J. and Russel, D.:** Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (2001).

18. **Mita, S., Suzuki-Fujii, K., and Nakamura, K.:** Sugar-inducible expression of a gene for β-amylase in *Arabidopsis thaliana*. Plant Physiol., **107,** 895-904 (1995).

19. **Liu, Y.G., Shirano, Y., Fukaki, H., Yanai, Y., Tasaka, M., Tabata, S., and Shibata, D.:** Complementation of plant mutants with large genomic DNA

fragments by a transformation-competent artificial chromosome vector accelerates positional cloning. Proc. Natl. Acad. Sci. USA, **96,** 6535-6540 (1999).

20. **Koyama, T., Ono, T., Shimizu, M., Jinbo, T., Mizuno, R., Tomita, K., Mitsukawa, N., Kawazu, T., Kimura, T., Ohmiya, K., and Sakka, K.:** Promoter of *Arabidopsis thaliana* phosphate transporter gene drives root-specific expression of transgene in rice. J. Biosci. Bioeng., **99,** 38-42 (2005).

21. **Matsuoka, K. and Nakamura, K.:** Propeptide of a precursor to a plant vacuolar protein required for vacuolar targeting. Proc. Natl. Acad. Sci. USA, **88,** 834-838 (1991).

22. **Clough, S.J. and Bent, A.F.:** Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J., **16,** 735-743 (1998).

23. **Saito, T., Niwa, Y., Ashida, H., Tanaka, K., Kawamukai, M., Matsuda, H., and Nakagawa, T.:** Expression of a gene for cyclophilin which contains an amino-terminal endoplasmic reticulum-targeting signal. Plant Cell Physiol., **40,** 77-87 (1999).

24. **Kato, A., Hayashi, M., and Nishimura, M.:** Oligomeric proteins containing N-terminal targeting signals are imported into peroxisomes in transgenic *Arabidopsis*. Plant Cell Physiol., **40,** 586-591 (1999).

25. **Salama, N.R., Yeung, T., and Schekman, R.W.:** The Sec13p complex and reconstitution of vesicle budding from the ER with purified cytosolic proteins. EMBO J., **12,** 4073-4082 (1993).

26. **Yang, Y.D., Elamawi, R., Bubeck, J., Pepperkok, R., Ritzenthaler, C., and Robinson, D.G.:** Dynamics of COPII vesicles and the Golgi apparatus in cultured *Nicotiana tabacum* BY-2 cells provides evidence for transient association of Golgi stacks with endoplasmic reticulum exit sites. Plant Cell, **17,** 1513-1531 (2005).

27. **Toyooka, K., Moriyasu, Y., Goto, Y., Takeuchi, M., Fukuda, H., and Matsuoka, K.:** Protein aggregates are transported to vacuoles by a macroautophagic mechanism in nutrient-starved plant cells. Autophagy, **2,** 96-106 (2006).

28. **Karthikeyan, A.S., Varadarajan, D.K., Mukatira, U.T., D'Urzo, M.P., Damsz, B., and Raghothama, K.G.:** Regulated expression of Arabidopsis phosphate transporters. Plant Physiol., **130,** 221-233 (2002).

29. **Kimura, T., Takeda, S., Kyozuka, J., Asahi, T., Shimamoto, K., and Nakamura, K.:** The presequence of a precursor to the δ-subunit of sweet potato mitochondrial F_1ATPase is not sufficient for the transport of β-glucuronidase (GUS) into mitochondria of tobacco, rice and yeast cells. Plant Cell Physiol., **34,** 345-355 (1993).

Figure legends

FIG. 1. Schematic illustration of pGWBs. The structures between the right and left border sequences of pGWBs are shown. (A) pGWBs with CaMV35S::HPT-NosT marker: pGWB1, simple cloning vector for complementation; pGWB2, for overexpression using CaMV35S promoter; pGWB3 and pGWB35, for promoter assay with GUS and LUC reporters, respectively; pGWB28 and pGWB29, for TAP analysis with independent promoter and CaMV35S promoter, respectively; pGWB4, pGWB7, pGWB10, pGWB13, pGWB16, pGWB19, pGWB22, pGWB25, pGWB40, and pGWB43, for C-fusion with independent promoter; pGWB5, pGWB8, pGWB11, pGWB14, pGWB17, pGWB20, pGWB23, pGWB26, pGWB41 and pGWB44, for C-fusion with CaMV35S promoter; and pGWB6, pGWB9, pGWB12, pGWB15, pGWB18, pGWB21, pGWB24, pGWB27, pGWB42, and pGWB45, for N-fusion with CaMV35S promoter. The tags and reporters are shown on the bottom. (B) pGWBs with NosP::HPT-NosT marker for C-fusion. The reporters and tag are indicted on the bottom. RB, right border; LB, left border; Cm^r, chloramphenicol-resistant marker (chloramphenicol acetyl transferase) used for selection in bacteria; *ccd*B, negative selection marker used in bacteria; 35S, CaMV35S promoter; GUS, *β*-glucuronidase (4); LUC, modified luciferase, luc+ (5); sGFP, synthetic green fluorescent protein with S65T mutation (6, 7); 6His, hexahistidine tag; FLAG, FLAG-tag (12); 3HA, triple HA tag (13); 4Myc and 10Myc, 4 and 10 repeats of Myc tag, respectively (13); GST, glutathione *S*-transferase (14); T7, T7-epitope tag (15); TAP, tandem affinity purification (16); EYFP, enhanced yellow fluorescent protein (8); ECFP, enhanced cyan fluorescent protein (8).

FIG. 2. Nucleotide and amino acid sequences of junctions after cloning by LR reaction. The sequences around the *att*B1 and *att*B2 sites (underlined) are also shown. N indicates the nucleotide derived from the entry clone and the corresponding amino acid is indicated by X. The start sites of GUS and sGFP are shown by an arrow in

pGWB3, pGWB4, and pGWB5. The amino acid LYI upstream of the *att*B1 region in pGWB6 was derived from the C-terminal of sGFP. The original C-terminal sequence of sGFP (LYK∗) was converted to LYI. The tag regions in pGWB7-45 were boxed.

FIG. 3. Expression of GUS fusion protein in BY-2 cells. (A) GFP fluorescence in BY-2 cells expressing sGFP-GUS fusion protein transformed with pGWB6-*GUS*. Bar: 50 μm. (B) Proteins (4 μg) prepared from BY-2 cells were immunoblotted with anti-GUS antibody. BY-2 cells transformed with pBI121 (lane 2, GUS), pGWB2-*GUS* (lane 3, GUS), pGWB6-*GUS* (lane 4, sGFP-GUS), pGWB8-*GUS* (lane 5, GUS-6xHis), pGWB11-*GUS* (lane 6, GUS-FLAG), pGWB12-*GUS* (lane 7, FLAG-GUS), pGWB15-*GUS* (lane 8, 3xHA-GUS), pGWB18-*GUS* (lane 9, 4xMyc-GUS), and pGWB20-*GUS* (lane 10, GUS-10xMyc) were analyzed. Lane M, molecular weight marker; lane 1, wild type. (C) Immunoblotting with anti-GFP antibody (lanes 4–6). BY-2 cells transformed with pBI121 (lanes 1 and 4, GUS), pGWB2-*GUS* (lanes 2 and 5, GUS), and pGWB6-*GUS* (lanes 3 and 6, sGFP-GUS) were analyzed. Immunoblotting with anti-GUS antibody was also performed in parallel (lanes 1–3). (D) Immnoblotting with anti-FLAG antibody (lanes 4–6). BY-2 cells transformed with pBI21 (lanes 1 and 4, GUS), pGWB11-*GUS* (lanes 2 and 5, GUS-FLAG), and pGWB12-*GUS* (lanes 3 and 6, FLAG-GUS) were analyzed. Lanes 1–3, immunoblotting with anti-GUS antibody. (E) Immunoblotting with anti-HA antibody. BY-2 cells transformed with pBI121 (lanes 1 and 4), pGWB2-*GUS* (lanes 2 and 5, GUS), and pGWB15-*GUS* (lanes 3 and 6, 3xHA-GUS) were analyzed. Lanes 1–3, immunoblotting with anti-GUS antibody. (F) Immunoblotting with anti-Myc antibody (lanes 4–6). BY-2 cells transformed with pBI121 (lanes 1 and 4, GUS), pGWB18-*GUS* (lanes 2 and 5, 4xMyc-GUS), and pGWB20-*GUS* (GUS-10xMyc) were analyzed. Lanes 1-3, immunoblotting with anti-GUS antibody. Black and white arrowheads indicate the position of native GUS and the GUS fusion protein,

respectively (B-F).

FIG. 4. Subcellular localization of ATSEC13 protein fused with sGFP. BY-2 cells transformed with pGWB5-*ATSEC13* (A, ATSEC13-sGFP) and pGWB6-*ATSEC13* (B, sGFP-ATSEC13) were viewed by fluorescence microscopy. (C) BY-2 cells expressing YFP-*Arabidopsis* cytochrome b5 fusion protein. Bars: 50 μm.

FIG. 5. Promoter analyses using GUS fusion constructs. Expression profiles of *PHT1* promoter (3888 bp)::*GUS* fusion in *A. thaliana* transformed with pGWB3-*PHT1* (A and C) and pGWB203-*PHT1* (B and D) under a low-Pi (0 μM) condition. No expression was observed in the root meristematic region. Bars: 5 mm (A, B); 100 μm (C, D).

TABLE 1. Synthetic oligonucleotides

Oligos	Sequence[a, b, c]
(Linker)	
Xho I linker	5′-CCTCGAGG-3′
Sac I linker	5′-CGAGCTCG-3′
(Adapter)	
Sac I-*Xba* I-*Sac* I	5′-CCCTCTAGAGGGAGCT-3′
Hin dIII-*Xba* I-*Hin* dIII	5′-AGCTTGGGTCTAGACCCA-3′
Xba I-*Hpa* I-*Aor* 51HI-*Sac* I	5′-CTAGAGTTAACAGCGCTTAGAGCT-3′
	5′-CTAAGCGCTGTTAACT-3′
Xba I-*Aor* 51HI-*Hpa* I-*Sac* I	5′-CTAGAATGAGCGCTGTTAACCTAGAGCT-3′
	5′-CTAGGTTAACAGCGCTCATT-3′
(Oligo for tag sequence)	
6xHis	5′-CATCATCATCATCATCAT-3′
	5′-ATGATGATGATGATGATG-3′
FLAG	5′-GACTACAAGGATGACGATGACAAG-3′
	5′-CTTGTCATCGTCATCCTTGTAGTC-3′
T7-epitope	5′-ATGGCTAGCATGACTGGTGGACAGCAAATGGGT-3′
	5′-ACCCATTTGCTGTCCACCAGTCATGCTAGCCAT-3′
(Primer for PCR)	
3xHA	5′-CTGAAGCTTCGTACGCTGCAG-3′
	5′-CTAGCGCTGCACTGAGCAGCGTAATCTG-3′
4-10xMyc	5′-CTGAAGCTTCGTACGCTGCAG-3′
	5′-CTAGCGCTACCGTTCAAGTCTTCCTCGGA –3′
GST	5′-ATGTCCCCTATACTAGGTTATTGG-3′
	5′-GGATCCACGCGGAACCAGATC-3′
TAP	5′-AAGAGAAGATGGAAAAAGAATTTCA-3′
	5′-TCAGGTTGACTTCCCCGCGGA-3′
LUC	5′-ATGGAAGACGCCAAAAACATAAAG-3′
	5′-TTACACGGCGATCTTTCCGCC-3′
EYFP (ECFP)	5′-ATGGTGAGCAAGGGCGAGGAG-3′
	5′-CTTGTACAGCTCGTCCATGCCGA-3′
GUS-att B1	5′-AAAAAGCAGGCTTTATGTTACGTCCTGTAGAAAC-3′
GUS-att B2	5′-AGAAAGCTGGGTATTGTTTGCCTCCCTGCTG-3′
ATSEC13-att B1	5′-aaaaagcaggctCCGACACTATGCCAGGTCAGAAGATTGAAAC-3'
ATSEC13-att B2	5′-agaaagctgggtAGACACTAGGCTCAACAGCAGTAACTTGTT-3'
att B1	5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3′
att B2	5′-GGGGACCACTTTGTACAAGAAAGCTGGGT-3′
PHT1 promoter	5′-CACCAAAAGGGGTAATGTGTAAAGGGCA-3′
	5′-TTCCTAGAGCTCTATAATCATACAACGCAA-3′

[a]Restriction sites are underlined.

[b]Parts of the *att*B sequences are in lower-case letters.

[c]Upper and lower sequences indicate the sense and antisense oligo nucleotides, respectively.

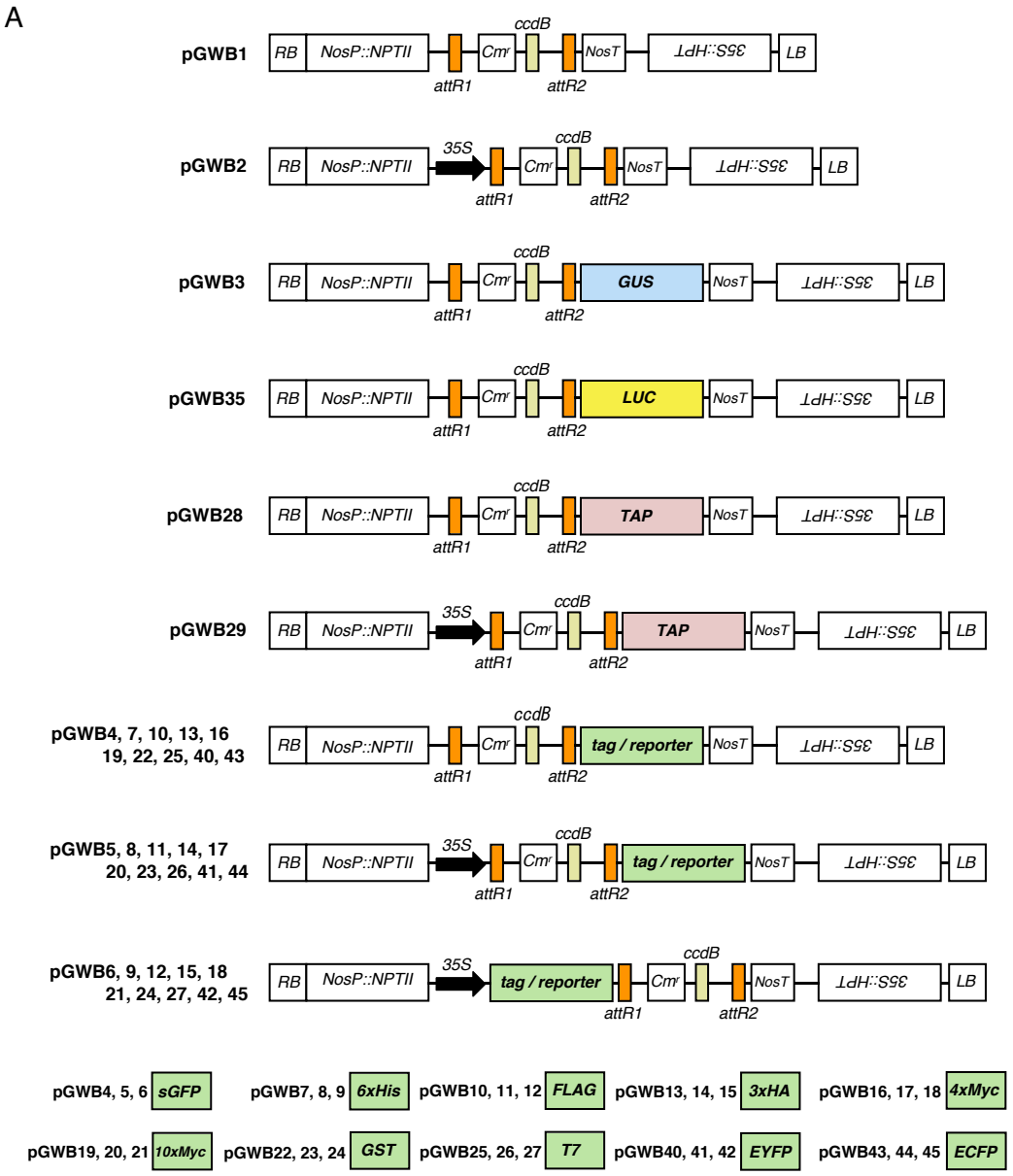


Fig. 1 (Nakagawa et al)

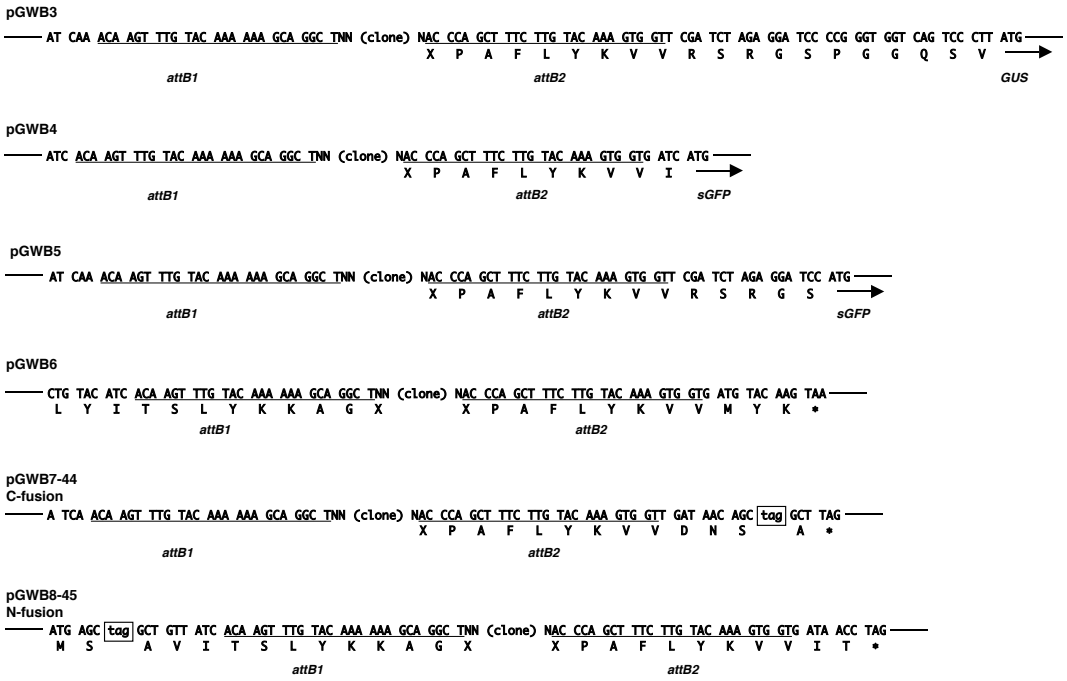
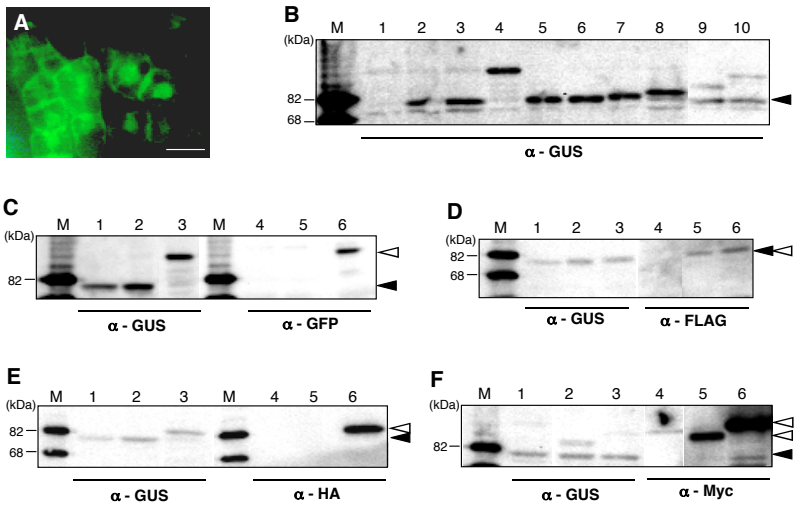


Fig. 2 (Nakagawa et al)



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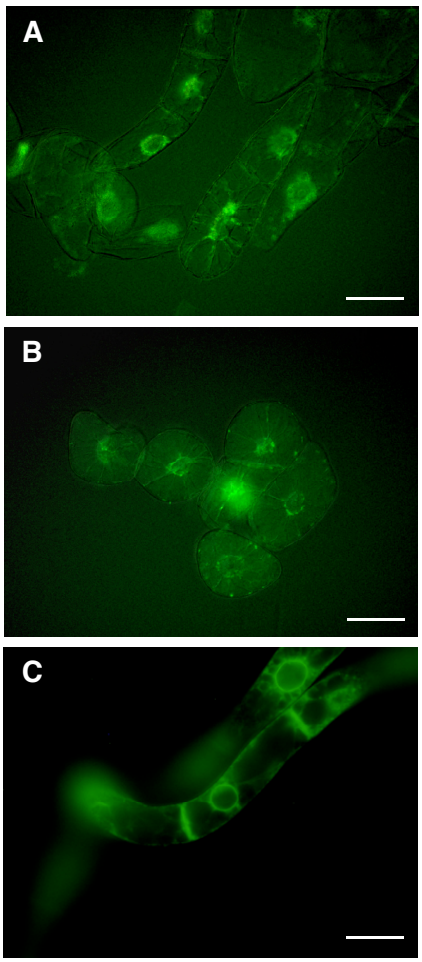

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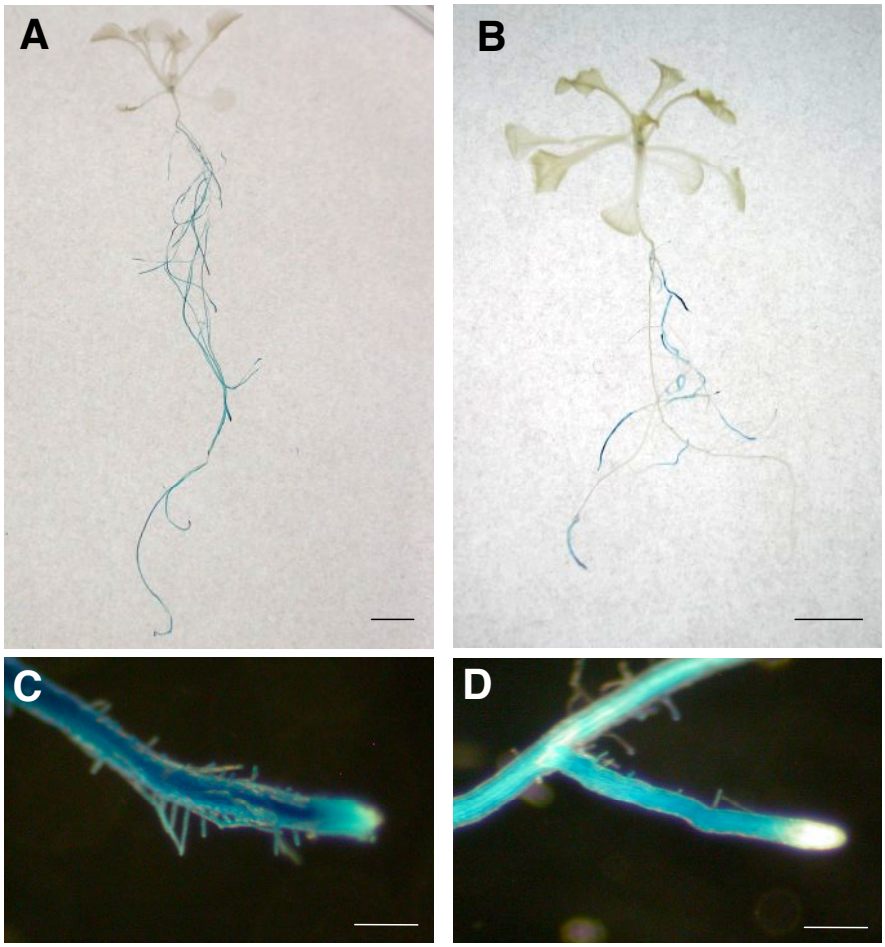

Fig. 5 (Nakagawa et al