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Communication

Improved Gateway Binary Vectors: High-Performance Vectors for Creation of Fusion Constructs in Transgenic Analysis of Plants

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We made a series of improved Gateway binary vectors (pGWBs) for plant transformation. Fifteen different reporters and tags, sGFP, GUS, LUC, EYFP, ECFP, G3GFP, mRFP, 6×His, FLAG, 3×HA, 4×Myc, 10×Myc, GST, T7, and TAP, were employed. Some vectors carry the 2×35S-Ω promoter for higher-level expression. The kanamycin- and hygromycin-resistant markers are independently available for each of the 43 types of vectors, thus an additional transformation of once-transformed plants can be carried out easily. Their small size and high-copy number in *Escherichia coli* make possible easier handling at plasmid preparation and sequencing. Improved pGWBs should be a powerful tool for transgenic research in plants.

Key words: binary vector; gateway cloning; reporter; tag

In plant gene studies, construction of fusion genes is indispensable for analysis of expression, protein localization, and protein-protein interactions *in vivo*. Fusion genes of promoter-GUS are routinely used in histochemical and quantitative expression analysis, while promoter-luciferase (LUC) fusions are preferable for expression analysis of rapidly responding genes. Recently, fluorescent proteins with different colors and properties were developed and applied for analysis of the dynamicity of protein localization, especially co-localization *in planta*. Many kinds of epitope tags are useful for co-purification and immuno-detection experiments during analysis of protein-protein interactions *in vivo*. We have constructed Gateway binary vectors (pGWBs)[1)] based on pBI, which make possible gene fusion to many reporters and tags through a simple and uniform procedure using Gateway cloning technology.[2)] Although useful, the previous pGWB remains unsatisfactory at some points. The most inconvenient feature of these vectors is the low yield of plasmids due to the low copy number in *E. coli*, which results in difficulty in verifying the nucleotide sequence after plasmid construction. Moreover, it is difficult to introduce additional

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Abbreviations: CaMV, cauliflower mosaic virus; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; FRET, fluorescence resonance energy transfer; HPT, hygromycin phosphotransferase; Hyg^r, hygromycin-resistant; Km^r, kanamycin-resistant; LUC, luciferase; mRFP, monomeric red fluorescent protein; NPTII, neomycin phosphotransferase II; Pnos, nopaline synthase promoter; sGFP, synthetic green fluorescent protein with S65T mutation; Spc^r, spectinomycin-resistant; TAP, tandem affinity purification; Tnos, nopaline synthase terminator

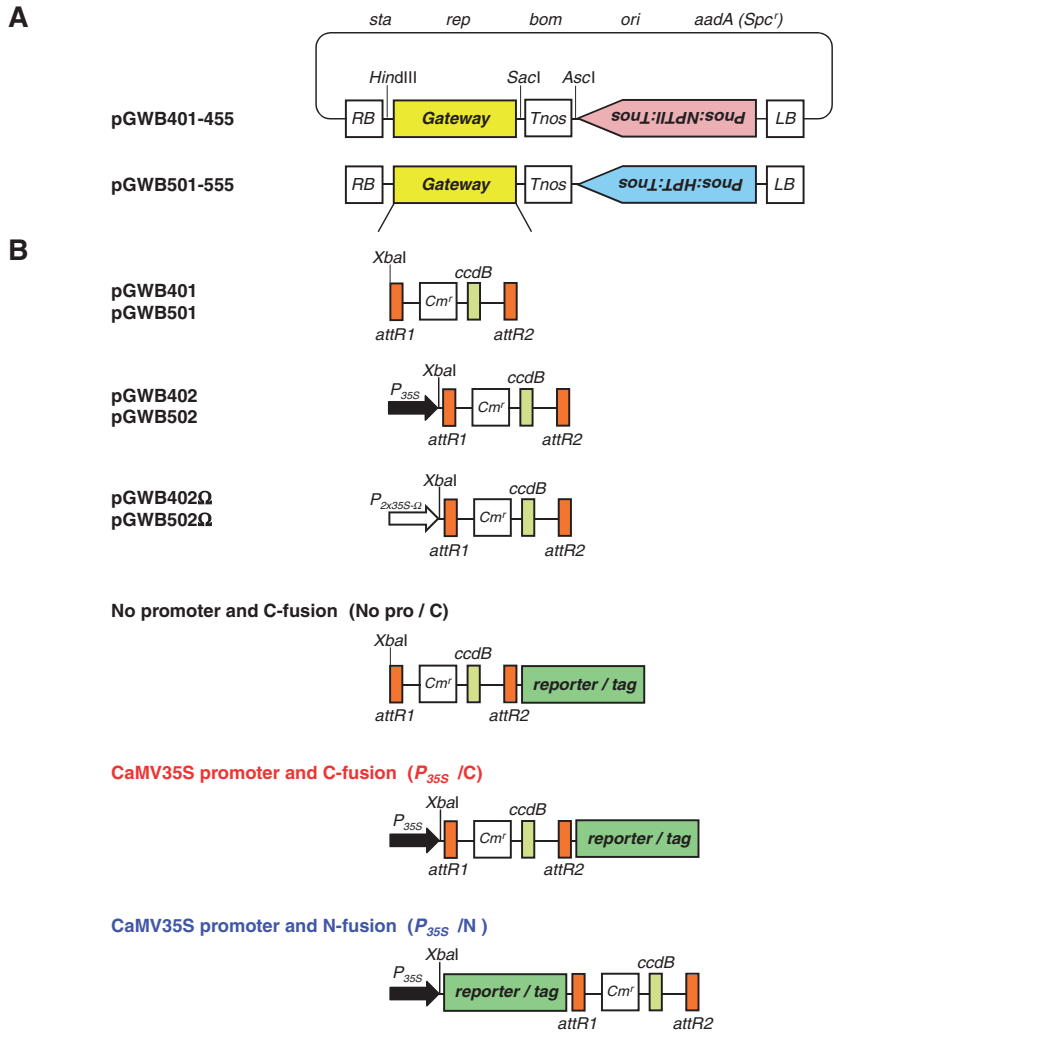
genes into plants once transformed by these pGWBs, because the T-DNA of the previous pGWBs contains both kanamycin-resistant (Km^r) and hygromycin-resistant (Hyg^r) markers, constructed with neomycin phosphotransferase II (NPTII) and hygromycin phosphotransferase (HPT) respectively. In this report, we describe new pGWBs improved to overcome the above disadvantages and to be more convenient and broadly applicable.

To construct the new pGWBs, plasmids were handled according to standard methods.[3] In PCR, KOD DNA polymerase (Toyobo, Tokyo) was used to make amplified product with blunt ends. All linker, adapters, and primers are listed in Supplemental Table 1; see *Biosci. Biotechnol. Biochem.* Web site. First, pPZP221[4] was modified to make backbone vectors (pGWB400 and pGWB500) for the new pGWB series. The *Sac*I-*Eco*RI fragment of pBI101 containing the nopaline synthase terminator (Tnos) was cloned into the *Sac*I-*Eco*RI sites of pPZP221. The Δ*Eco*RI adapter was introduced into the *Eco*RI site of the resulting plasmid to destroy the *Eco*RI site, then the gentamycin-resistant marker was removed by *Psh*BI digestion and replaced with an *Eco*RI linker. The *Eco*RI-*Asc*I-*Swa*I-*Eco*RI adapter was introduced into the newly made *Eco*RI site in the orientation described here. The resulting plasmid was designated pPZP-NosT-*Asc*I/*Swa*I. The selection markers, driven by nopaline synthase promoter (Pnos), were introduced as follows: For the Km^r marker, the *Pnos:NPTII:Tnos* sequence was prepared by PCR using pBI101 as a template with NPTII primers. For the Hyg^r marker, the *Pnos:HPT:Tnos* sequence was prepared by PCR using pLYTAC7[5] as a template with HPT primers. These marker fragments were cloned into the *Swa*I site of pPZP-NosT-*Asc*I/*Swa*I in reverse orientation, and the resulting plasmids were designated pGWB400 (*Pnos:NPTII:Tnos*) and pGWB500 (*Pnos:HPT:Tnos*) respectively. Media containing 100 mg/l spectinomycin were used to select *E. coli* harboring these plasmids.

Most of the intermediate plasmids (pUGWs) containing the Gateway cassette and reporter/tag genes, which were used to construct the previous pGWBs,[1] were reused for new series of pGWBs. Additional pUGWs were constructed as described in the Supplemental Text; see *Biosci. Biotechnol. Biochem.* Web site. The *Hin*dIII-*Sac*I or *Xba*I-*Sac*I fragments of pUGW1–55 were introduced into the same site of pGWB400 and pGWB500 to make pGWB401–455 and pGWB501–555 respectively. *E. coli* (strain DB3.1) harboring the new pGWBs was selected on media containing 100 mg/l spectinomycin and 30 mg/l chloramphenicol.

The structures of the new pGWBs are shown in Fig. 1. The pGWB400 series carry a Km^r marker (*Pnos:NPTII:Tnos*), and the pGWB500 series carry a Hyg^r marker (*Pnos:HPT:Tnos*). Both markers were placed in reverse orientation to the gene cloned by LR reaction. The last two digits of the plasmid name indicate the structures around the Gateway cassette, which are common to new and previous pGWBs. pGWB401 and 501 are simple cloning vectors with neither promoters nor reporters/tags, usable for complementation experiments with genomic fragments. pGWB402 and pGWB502 carry a cauliflower mosaic virus (CaMV) 35S promoter[6] for constitutive expression in plants. pGWB402 Ω and 502 Ω are suitable for highly increased expression of protein-coding genes, because they carry a modified CaMV35S promoter in which the enhancer regions are duplicated, along with the Ω translational enhancer from tobacco mosaic virus[7] (2×35S-Ω). pGWB433 and pGWB533 are suitable for promoter analysis with GUS reporter. The long 5′-UTR attached to the GUS gene of previous pGWBs (*i.e.*, pGWB3 and pGWB203) was shortened for enhanced expression in plants. pGWB435 and pGWB535 were constructed for promoter-LUC assay. pGWB404–406, pGWB440–455, pGWB504–506, and pGWB540–555 are suitable for expressing proteins *in planta* as protein-fusions with fluorescent reporters (sGFP, G3GFP, EYFP, ECFP, and mRFP). sGFP is a synthetic GFP with the S65T mutation.[8] G3GFP,[9] a brighter variant of GFP with S65A/Y145F mutations, made it possible to visualize weakly expressed gene products in plants. mRFP (monomeric red fluorescent protein),[10] a monomer variant of the DsRed,[11] facilitated the analysis of co-localization in combination with GFP. The EYFP (enhanced yellow fluorescent protein) and ECFP (enhanced cyan fluorescent protein) were derivative of EGFP (enhanced GFP)[12] and applicable in FRET (fluorescence resonance energy transfer) analysis.[13] Three fusion types are available for each reporter, namely, the vectors for the constitutive expression of proteins that fused with reporters at their C-terminus (CaMV35S promoter and C-fusion), those for the constitutive expression of proteins that fused with reporters at their N-terminus (CaMV35S promoter and N-fusion), and those for expression of C-fusion proteins by their own promoters (no promoter and C-fusion). These promoter-less vectors are also available for promoter analysis. pGWB407–429 and pGWB507–529 are suitable for expression of proteins labeled with various epitope tags (6×His, FLAG, 3×HA, 4×Myc, 10×Myc, GST, T7, and TAP: see figure legend for details). The tandem affinity purification (TAP)[14] tag is available as C-fusion vectors with or without the CaMV35S promoter. Three types of vectors (no promoter and C-fusion, CaMV35S promoter and C-fusion, and CaMV35S promoter and N-fusion) are available for others.

For all vectors, the sequences of PCR amplified regions and ligation junctions were confirmed by sequencing. We also sequenced the remaining backbone of new pGWBs and found a slight discrepancy between our plasmids and the recorded pPZP221 in the database. The complete nucleotide sequence of new pGWBs appears in GenBank/EMBL/DDBJ databases under accession nos. AB294425 to AB294510. pGWB402 Ω and pGWB502 Ω were registered as pGWB402omega and pGWB502omega respectively. The linker sequences around the *att*B1 and *att*B2 sites are shown in Sup-



C

	sGFP	6xHis	FLAG	3xHA	4xMyc	10xMyc	GST	T7
No pro / C	pGWB 404, 504	407, 507	410, 510	413, 513	416, 516	419, 519	422, 522	425, 525
P_{35S} / C	pGWB 405, 505	408, 508	411, 511	414, 514	417, 517	420, 520	423, 523	426, 526
P_{35S} / N	pGWB 406, 506	409, 509	412, 512	415, 515	418, 518	421, 521	424, 524	427, 527

	TAP	GUS	LUC	EYFP	ECFP	G3GFP	mRFP
No pro / C	pGWB 428, 528	433, 533	435, 535	440, 540	443, 543	450, 550	453, 553
P_{35S} / C	pGWB 429, 529			441, 541	444, 544	451, 551	454, 554
P_{35S} / N				442, 542	445, 545	452, 552	455, 555

Fig. 1. Schematic Illustration of the Improved pGWBs.

A, Outline of new pGWBs. pGWB401–455 and pGWB501–555 contain Km^r and Hyg^r markers, respectively, that are placed in reverse orientation to the genes cloned by LR reaction. B, The structures of the region indicated as "*Gateway*" in (A). Only the general structures are shown in the case of vectors for the fusion construct; *i.e.*, no promoter and C-fusion (No pro/C), CaMV35S promoter and C-fusion (P_{35S}/C), CaMV35S promoter and N-fusion (P_{35S}/N). C, Reporters and tags employed in the new pGWBs illustrated in (B). The vector number corresponds to the combination of reporter/tag and fusion type. sGFP, synthetic green fluorescent protein with S65T mutation;[8,17] 6×His, hexahistidine tag; FLAG, FLAG-tag;[18] 3×HA, triple HA tag;[19] 4×Myc and 10×Myc, 4 and 10 repeats of the Myc tag respectively;[19] GST, glutathione *S*-transferase;[20] T7, T7-epitope tag;[21] TAP, tandem affinity purification;[14] GUS, *β*-glucuronidase;[22] LUC, modified luciferase, luc+;[23] EYFP, enhanced yellow fluorescent protein;[13] ECFP, enhanced cyan fluorescent protein;[13] G3GFP, G3 green fluorescent protein;[9] mRFP, monomeric red fluorescent protein.[10] *RB*, right border; *LB*, left border. *sta*, region for atability in *Agrobacterium*; *rep*, broad host-range replication origin; *bom*, *cis*-acting element for conjugational transfer; *ori*, ColE1 replication origin. *Cm^r*, chloramphenicol-resistant marker (chloramphenicol acetyl transferase) used for selection in the bacteria; *aadA*, spectinomycin-resistant (Spc^r) marker used for selection in the bacteria; *ccd*B, negative selection marker used in the bacteria.

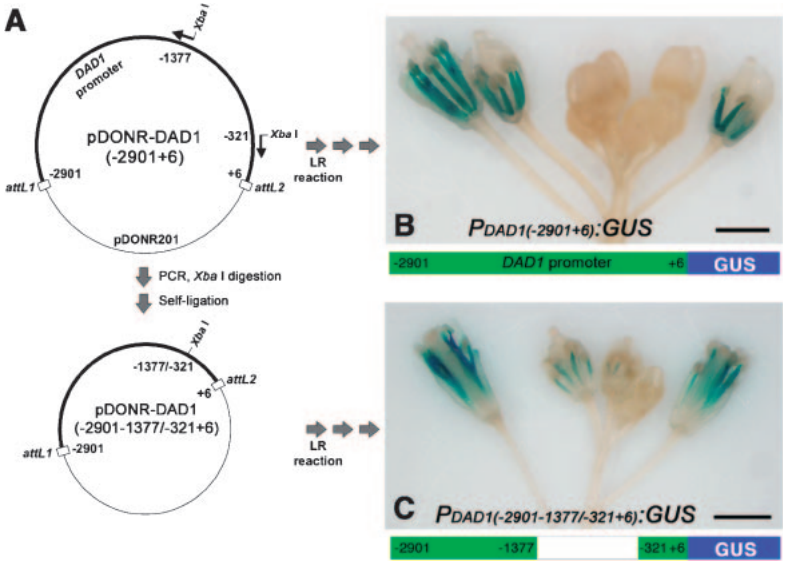


Fig. 2. Promoter Analysis of the *DAD1* Gene.

A, Structures of an entry clone that contained a 2.9-kb fragment of *DAD1* promoter (top), and its truncated derivative produced by PCR and self-ligation (bottom). Two PCR primers that possessed *Xba* I sites at their 5′ termini are indicated by solid arrows. The *Xba* I site was inserted for easier construction, but generally it is not essential. Thick and thin lines drawing the plasmids indicate part of the *DAD1* promoter and part of the pDONR201 vector respectively. The length between the *att*L1 and *att*L2 sites of the vector portion was 2.3 kb. B and C, Histochemical analysis of GUS expression in the inflorescences of Arabidopsis plants possessing the $P_{DAD1(-2901+6)}$*:GUS* gene (B) and the $P_{DAD1(-2901-1377/-321+6)}$*:GUS* gene (C) respectively. Bars = 1 mm.

plemental Fig. 1; see *Biosci. Biotechnol. Biochem.* Web site.

These pPZP-based new pGWBs are much more advantageous than the pBI-based previous ones for the following reasons: First, the new pGWBs are smaller in size and their copy numbers in *E. coli* are much higher.[4] This alteration allows easy handling in cloning experiments. For example, the efficiency of the LR reaction with the new pGWBs was much higher than that of the previous ones, and accordingly it is not necessary to linearize them prior to the LR reaction. Furthermore, clear signals were detected during sequence verification of the constructed plasmids. Second, all types of vectors with the Km^r marker (pGWB400 series) and Hyg^r marker (pGWB500 series) are independently available, and so it was easy to introduce a second gene into plants that have been transformed by these vectors. For example, plants transformed with pGWB4xx can easily be re-transformed with pGWB5xx vectors. In the case of the previous pGWBs, a third selection marker was required for re-transformation experiments because they carried both the Km^r and the Hyg^r marker on one vector. As a result of these improvements, the new pGWBs are applicable over a broad range of transgenic experiments and should thus accelerate plant research.

To test the performance of these vectors, we made a promoter:GUS construct using pGWB433. A promoter fragment spanning the sequence between −2901 and +6 (the A of the translational initiation codon was designated +1) of the Arabidopsis *DEFECTIVE IN ANTHER DEHISCENCE1* (*DAD1*) gene was prepared from the *DAD1* genomic clone pGDAD1-HS[15] by PCR with DAD1−2901*att*B1 and DAD1 + 6*att*B2 primers. The amplified fragment was cloned into pDONR201 by the Gateway BP reaction to produce an entry clone, pDONR-DAD1(−2901+6) (Fig. 2A). The entry clone was then used in the LR reaction with the closed circular form of pGWB433 to create a binary plasmid containing the $P_{DAD1(-2901+6)}$*:GUS* gene. The BP and LR reactions were performed as described in the manufacturer's manual (Invitrogen, Carlsbad, CA). Most *E. coli* transformants grown on a medium containing 100 mg/l spectinomycin harbored the accurately recombined plasmids. The plasmids were introduced into *Agrobacterium tumefaciens* strain C58C1 (pMP90), and bacteria resistant to 100 mg/l spectinomycin were used for the transformation of Arabidopsis (Col-0 accession) by a floral dip method.[16] More than 1% of the T1 seeds germinated and grew on a selection plate containing 30 to 50 mg/l kanamycin and 100 mg/l carbenicillin (kanamycin should be replaced by 10 to 20 mg/l hygromycin B when the pGWB500 series is used). GUS expression was specifically observed in stamen filaments of developed flower buds and opened flowers (Fig. 2B), which is consistent with a previous study in which a longer promoter fragment was used.[15]

To identify the *cis*-regulatory elements for gene expression, promoter analyses with serially truncated promoters are a widely used strategy. The Gateway system is best suited to this purpose, because a promoter fragment cloned on a small entry vector can easily be truncated by a simple PCR amplification. We carried out PCR using two primers, DAD1−1377-*Xba*I-R and DAD1−321-*Xba*I, and a template entry clone pDONR-

DAD1(−2901+6) (Fig. 2A). The PCR product was self-ligated after *Xba* I digestion, then introduced into *E. coli* for propagation. The new entry clone created, pDONR-DAD1(−2901−1377/−321+6), was used in the LR reaction with pGWB433, and the resulting binary plasmid containing the $P_{DAD1(-2901-1377/-321+6)}$*:GUS* gene was utilized in plant transformation. Again we observed stamen-specific GUS expression in transgenic plants (Fig. 2C), and hence concluded that the *cis*-elements for stamen-specific expression are localized between −2901 and −1377 and/or beside the TATA region (between −321 and +6) of the *DAD1* promoter.

In conclusion, we reconstructed pGWBs by replacing their backbone vector, deleting one of two selection markers on a vector, and adding new reporters and a promoter in the line-up. These improvements made the new pGWBs more useful than the previous ones, and should therefore aid molecular biological analysis in plant gene research and biotechnology. All vectors described in this report will be available for non-commercial research purposes, although the permission of the original developers will be required for some reporters.

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References

1) Nakagawa, T., Kurose, T., Hino, T., Tanaka, K., Kawamukai, M., Niwa, Y., Toyooka, K., Matsuoka, K., Jinbo, T., and Kimura, T., Development of series of Gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. *J. Biosci. Bioeng.*, **104**, 34–41 (2007).
2) 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).
3) Sambrook, J., and Russell, D., "Molecular Cloning, a Laboratory Manual" 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor (2001).
4) Hajdukiewicz, P., Svab, Z., and Maliga, P., The small, versatile *pPZP* family of *Agrobacterium* binary vectors for plant transformation. *Plant Mol. Biol.*, **25**, 989–994 (1994).
5) 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).
6) Fang, R. X., Nagy, F., Sivasubramaniam, S., and Chua, N. H., Multiple *cis* regulatory elements for maximal expression of the cauliflower mosaic virus 35S promoter in transgenic plants. *Plant Cell*, **1**, 141–150 (1989).
7) Gallie, D. R., Posttranscriptional regulation of gene expression in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **44**, 77–105 (1993).
8) 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).
9) Kawakami, S., and Watanabe, Y., Use of green fluorescent protein as a molecular tag of protein movement *in vivo*. *Plant Biotechnol.*, **14**, 127–130 (1997).
10) Campbell, R. E., Tour, O., Palmer, A. E., Steinbach, P. A., Baird, G. S., Zacharias, D. A., and Tsien, R. Y., A monomeric red fluorescent protein. *Proc. Natl. Acad. Sci. USA*, **99**, 7877–7882 (2002).
11) Matz, M. V., Fradkov, A. F., Labas, Y. A., Savitsky, A. P., Zaraisky, A. G., Markelov, M. L., and Lukyanov, S. A., Fluorescent proteins from nonbioluminescent Anthozoa species. *Nat. Biotechnol.*, **17**, 969–973 (1999).
12) Yang, T. T., Cheng, L., and Kain, S. R., Optimized codon usage and chromophore mutations provide enhanced sensitivity with the green fluorescent protein. *Nucleic Acids Res.*, **24**, 4592–4593 (1996).
13) 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).
14) 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).
15) Ishiguro, S., Kawai-Oda, A., Ueda, J., Nishida, I., and Okada, K., The *DEFECTIVE IN ANTHER DEHISCENCE1* gene encodes a novel phospholipase A1 catalyzing the initial step of jasmonic acid biosynthesis, which synchronizes pollen maturation, anther dehiscence, and flower opening in Arabidopsis. *Plant Cell*, **13**, 2191–2209 (2001).
16) 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).
17) Niwa, Y., A synthetic green fluorescent protein gene for plant biotechnology. *Plant Biotechnol.*, **20**, 1–11 (2003).
18) 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).
19) Bähler, J., Wu, J. Q., Longtine, M. S., Shah, N. G., McKenzie, A., III, 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).
20) 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).
21) 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).

22) 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).

23) Sherf, B. A., and Wood, K. V., Firefly luciferase engineered for improved genetic reporting. *Promega Notes*, **49**, 14–21 (1994).