Gateway Binary Vectors with Organelle-Targeted Fluorescent Proteins for Highly Sensitive Reporter Assay in Gene Expression Analysis of Plants

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Highlights

- Rapid and efficient cloning of a promoter of interest in a binary vector with the commonly used entry clones, *att*L1-promoter-*att*L2 and *att*L4-promoter-*att*R1.
- ER-, nucleus-, peroxisome-, and mitochondria-targeted sGFPs (ER-, NLS-, Px-, and Mt-sGFP) and nucleus-, peroxisome-, and mitochondria-targeted TagRFPs (NLS-, Px-, and Mt-TagRFP) are available for promoter:reporter analysis in plants.
- The system is equipped with four kinds of plant selection markers (kanamycin-, hygromycin-, BASTA-, and tunicamycin-resistance) for various transformation purposes.
- Brighter fluorescence signals were successfully detected by promoter:Px-sGFP and promoter:NLS-sGFP, indicating the availability of the developed cloning system for highly sensitive promoter assays.

Abstract

Fluorescent proteins are valuable tools in the bioscience field especially in subcellular localization analysis of proteins and expression analysis of genes. Fusion with organelle-targeting signal accumulates fluorescent proteins in specific organelles, increases local brightness, and highlights the signal of fluorescent proteins even in tissues emitting a high background of autofluorescence. For these advantages, organelle-targeted fluorescent proteins are preferably used for promoter:reporter assay to define organ-, tissue-, or cell-specific expression pattern of genes in detail. In this study, we have developed a new series of Gateway cloning technology-compatible binary vectors, pGWBs (*att*R1-*att*R2 acceptor sites) and R4L1pGWB (*att*R4-*att*L1 acceptor sites), carrying organelle-targeted synthetic green fluorescent protein with S65T mutation (sGFP) (ER-, nucleus-, peroxisome-, and mitochondria-targeted sGFP) and organelle-targeted tag red fluorescent protein (TagRFP) (nucleus-, peroxisome-, and mitochondria-targeted TagRFP). These are available for preparation of promoter:reporter constructs by an LR reaction with a promoter entry clone *att*L1-promoter-*att*L2 (for pGWBs) or *att*L4-promoter-*att*R1 (for R4L1pGWBs), respectively. A transient expression experiment with particle bombardment using cauliflower mosaic virus 35S promoter-driven constructs has confirmed the correct localization of newly developed organelle-targeted TagRFPs by a co-localization analysis with the previously established organelle-targeted sGFPs. More intense and apparent fluorescence signals were detected by the nucleus- and peroxisome-targeted sGFPs than by the normal sGFPs in the promoter assay using transgenic *Arabidopsis thaliana*. The new pGWBs and R4L1pGWBs developed here are highly efficient and may serve as useful platforms for more accurate observation of GFP and RFP signals in gene expression analyses of plants.

Keywords

promoter assay; organelle-targeted fluorescent protein; binary vector; plant transformation; Gateway cloning.

1. Introduction

Fluorescent proteins are the versatile probes in live imaging and become an essential tool for cell biology and physiology. In addition to the normal types, fluorescent proteins fused with organelle-targeting signals were widely used for analysis of subcellular localization of proteins and organelle dynamics in living organisms including plants. Experiments using GFP fused with peroxisome targeting signal 1 (PTS1) or PTS2 revealed that directional movement of peroxisomes depends on actin filaments in *Arabidopsis thaliana* (Jedd and Chua, 2002; Mano et al., 2002). Analysis using GFP fused with mitochondrial presequences from *A. thaliana* CPN60, *Nicotiana plumbaginifolia* β-ATPase, and yeast COX 4p showed different mobility and shape of mitochondria in plant cells depending on the location, developmental stage, and physiological conditions (Lo et al., 2004; Logan and Leaver, 2000). GFP fused with a signal sequence and an ER retention signal was used to analyze the ER body, a characteristic structure derived from ER under stress conditions, in *A. thaliana* (Matsushima et al., 2002). Because the accumulation of fluorescent proteins in organelles brings brighter and distinctive signals, organelle-targeted fluorescent proteins were also used in promoter:reporter experiments for visualization of target tissues or cells by a specific promoter, and for precise expression analysis of unidentified promoters. Usually, nucleus-targeted fluorescent proteins are used for these purposes and many plant promoters were analyzed by nucleus-targeted GFP (Goh et al., 2012; Ortiz-Morea et al., 2016).

Recently, Gateway cloning has become one of the most widely used techniques to clone DNA fragments into vectors for many research fields including plants (Dalal et al., 2015; Earley et al., 2006; Ishizaki et al., 2015; Karimi et al., 2002; Wang et al., 2013; Zhong et al., 2008). In the Gateway cloning technology, two types of promoter entry clones are mostly used for different cloning strategies. The *att*L1-promoter-*att*L2 entry clone is used to prepare simple constructs such as promoter:reporter (*att*B1-promoter-*att*B2-reporter), whereas, the *att*L4-promoter-*att*R 1 entry clone is usually used for combinatorial cloning with *att*L1-cDNA-*att*L2 to prepare promoter:cDNA-reporter (*att*B4-promoter-*att*B1-cDNA-*att*B2-reporter) constructs. Previously, we have developed series of Gateway binary vectors (pGWBs) compatible with Gateway cloning technology for construction of fusions with many kinds of tags, epitopes, β-glucuronidase (GUS), luciferase (LUC), and fluorescent proteins including synthetic green fluorescent protein with S65T mutation (sGFP), enhanced yellow fluorescent protein (EYFP), enhanced cyan fluorescent protein (ECFP), brighter variant of GFP with S65A/Y145F mutations (G3GFP), monomeric red fluorescent protein (mRFP) and tag red fluorescent protein (TagRFP) (Nakagawa et al., 2009). In addition to the pGWB series carrying *att*R1-*att*R2 sites for LR reaction with an *att*L1-promoter-*att*L2 entry clone (Nakagawa et al., 2007a; Nakagawa et al., 2007b; Nakamura et al., 2010; Tanaka et al., 2013), we also developed R4L1pGWB series carrying *att*R4-*att*L1 acceptor sites specialized for creation of promoter:reporter constructs by an LR reaction with *att*L4-promoter-*att*R1 entry clone (Nakamura et al., 2009; Tanaka et al., 2011). These vectors have been frequently used in promoter assays of transgenic plants by microscopic observation of visible reporters, GUS, and fluorescent proteins to determine the organs, tissues, and cells expressing a gene of interest in detail.

In this study, we developed new pGWBs (*att*R1-*att*R2 acceptor sites) and R4L1pGWBs (*att*R4-*att*L1 acceptor sites) carrying organelle-targeted sGFPs (ER-, nucleus-,

peroxisome-, and mitochondria-targeted sGFPs) and organelle-targeted TagRFPs (nucleus-, peroxisome-, and mitochondria-targeted TagRFPs) to facilitate promoter:reporter assays in plants. We also tested the performance of these vectors and reported the highly sensitive detection of fluorescence signals with nucleus- and peroxisome-targeted sGFP compared with normal sGFP (no organelle-targeted type) in promoter:reporter analysis using transgenic plants.

2. Materials and Methods

2.1. Plasmid constructions

Plasmids were constructed according to standard methods (Sambrook and Russell, 2001). KOD DNA polymerase (Toyobo, Osaka, Japan) was used for PCR to prepare amplified products with blunt ends. The sequences of the PCR-amplified regions and ligation junctions were confirmed by sequence analysis. All primers used in this study are listed in Supplemental Table S1. Synthetic green fluorescent protein (sGFP) (Chiu et al., 1996) was amplified using pGWB404 (Nakagawa et al., 2007a) as a template with the primers sGFP-F and sGFP-R. Amplified DNA was introduced into R4L1pUGW1 (Nakamura et al., 2009) to make R4L1pUGW4 (R4-L1-sGFP). ER-targeted sGFP (ER-sGFP) sequence was amplified using pNMG3 (the signal peptide of *A. thaliana* endo-xyloglucan transferase plus sGFP hooked with the HDEL retention signal at the C terminus) (Takeuchi et al., 2000) as a template with the primers ER-sGFP-F and ER-sGFP-R. Nucleus-targeted sGFP (NLS-sGFP, carrying nuclear localization sequence PKKKRKV at N-terminal region) was amplified using 35SΩ-NLS-sGFP(S65T) (Chiu et al., 1996) as a template with the primers NLS-sGFP-F and sGFP-R. Peroxisome-targeted sGFP (Px-sGFP, carrying peroxisome-targeting sequence SKL

at C-terminal) was amplified using pGWB404 as a template with the primers sGFP-F and Px-sGFP-R. Nucleus-targeted TagRFP (NLS-TagRFP, carrying PKKKRKV at N-terminal region) was amplified using pGWB659 (Nakamura et al., 2010) as a template with the primers NLS-TagRFP-F and TagRFP-R. Peroxisome-targeted TagRFP (Px-TagRFP, carrying SKL at C-terminal) was amplified using a pGWB659 as a template with the primers TagRFP-F and Px-TagRFP-R. Mitochondria-targeted sGFP and TagRFP (Mt-sGFP and Mt-TagRFP), carrying N-terminal 57 amino acids of *A. thaliana* F_1 ATPase γ subunit (Lee et al., 2012) were synthesized by GeneArt Gene Synthesis (Thermo Fisher Scientific, Kanagawa, Japan). These DNA fragments were introduced into Aor51HI site of pUGW2 (Nakagawa et al., 2007a) and R4L1pUGW1 (Nakamura et al., 2009) to make pUGW62 (R1-R2-ER-sGFP), pUGW65 (R1-R2-NLS-sGFP), pUGW68 (R1-R2-Px-sGFP), pUGW71 (R1-R2-Mt-sGFP), pUGW85 (R1-R2-NLS-TagRFP), pUGW88 (R1-R2-Px-TagRFP), pUGW91 (R1-R2-Mt-TagRFP), R4L1pUGW62 (R4-L1-ER-sGFP), R4L1pUGW65 (R4-L1-NLS-sGFP), R4L1pUGW68 (R4-L1-Px-sGFP), R4L1pUGW71 (R4-L1-Mt-sGFP), R4L1pUGW85 (R4-L1-NLS-TagRFP), R4L1pUGW88 (R4-L1-Px-TagRFP), and R4L1pUGW91 (R4-L1-Mt-TagRFP), respectively. The DNA fragments containing *att*R1-Cm^r-*ccd*B-*att*R2-reporter prepared from resulting pUGWs and DNA fragments containing *att*R4-Cm^r-*ccd*B-*att*L1-reporter prepared from resulting R4L1pUGWs were introduced into pGWB400, pGWB500 (Nakagawa et al., 2007b), pGWB600 (Nakamura et al., 2010) and pGWB700 (Tanaka et al., 2011) for construction of destination vectors pGWBs and R4L1pGWBs indicated in Fig.1. The Cm^r is the chloramphenicol resistance and *ccd*B is the *control of cell death* used as a negative selection marker in *Escherichia coli*. Transformed *E. coli* DB3.1 (Thermo Fisher) were selected on Luria broth (LB) media containing appropriate antibiotics (30 mg/L of chloramphenicol, 50 mg/L of ampicillin, or 100 mg/L of spectinomycin).

2.2. Preparation of entry clones and expression constructs

The DNA fragment of the cauliflower mosaic virus 35S promoter was amplified using pGWB402 (Nakagawa et al., 2007a) as a template with the primers 35Spro-attB4F and 35Spro-attB1R. The DNA fragment of 3-ketoacyl-CoA thiolase 2 (KAT2, AT2G33150) promoter spanning -2074 to +6 (A of ATG is +1) was amplified using genomic DNA of *A. thaliana* (Col-0 accession) as a template with the primers KAT2pro-GWB4F and KAT2pro-GWB1R. These DNA fragments were applied for second PCR with the primers attB4-adapt and attB1R-adapt, and introduced into pDONR P4-P1R (Thermo Fisher) by BP reaction to make *att*L4-Pro35S-*att*R1 and *att*L4-ProKAT2-*att*R1 entry clones. The DNA fragment of DAD1-LIKE LIPASE2 (DALL2, AT1G51440) promoter spanning -2413 to +10 was amplified using genomic DNA of *A. thaliana* (Col-0 accession) with DAL2-2413FattB4 and DAL2-21RattB1 primers, and introduced into pDONR P4-P1R by BP reaction to make an *att*L4-ProDALL2-*att*R1 entry clone. The *att*L1-ProMYB21:MYB21-*att*L2 entry clone carrying promoter and coding sequence of MYB21 (AT3G27810) was prepared as described in Reeves et al. (2012). The sequences between *att* sites of obtained entry clones were confirmed by sequence analysis. Transfer of the DNA fragment from entry clones to pGWB or R4L1pGWB was performed by LR reaction according to the manufacturer's instruction.

2.3. Transient expression analysis using Japanese leek

Two expression constructs were mixed at 1:1 w/w ratio and introduced into Japanese leek epidermal cells using particle bombardment technique as described in Hino et al. (2011).

2.4. Generation of transgenic A. thaliana for stable expression analyses

Transformation of *Agrobacterium tumefaciens* C58C1 (pMP90) was carried out using the freeze-thaw method (Weigel and Glazebrook, 2002). Transformation of *A. thaliana* (Col-0 accession) was performed by floral inoculating protocol (Narusaka et al., 2010) and inoculated plants were grown at 22 °C under long day photoperiod (16 hr light /8 hr dark cycle). Harvested T0 seeds were vernalized at 4 °C for 2-3 days and grown on Murashige and Skoog (MS) agar medium containing kanamycin (30 mg/L) or hygromycin (20 mg/L) or BASTA (0.0054 %) with Cefotax (100 mg/L) (Chugai Pharmaceutical Co., Tokyo, Japan) at 22 °C under continuous light conditions. Two-week-old kanamycin- or hygromycin-resistant seedlings (T1) were transplanted to Jiffy-7 (Jiffy Preforma Production K. K, Yokohama, Japan) and grown at 22 °C under long-day photoperiod. Ten-day-old BASTA-resistant seedlings (T1) were transferred to BASTA- and Cefotax-free plates to enhance root elongation. After 7 days, the seedlings were transplanted to Jiffy-7 and grown at 22 °C under long-day photoperiod. Homozygous T3 plants harboring transgene were used for expression analyses.

2.5. Expression analyses by confocal microscopy and measurement of fluorescence intensity

Fluorescence signals were examined with a TCS SP5 confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany) using an HCX IRAPO L 25.0×0.95 water-immersion objective lens. sGFP was excited with the argon laser line (488 nm) and TagRFP was excited with helium-neon laser line (543 nm). The fluorescence from sGFP and TagRFP were detected at 500-530 nm and 555-615 nm, respectively. The images were

obtained using the sequential scanning mode with a resolution of 1024×256 pixels with bidirectional scanning mode at speed of 200 Hz for transient expression analysis and 512×512 pixels at the speed of 400 Hz for stable expression analysis. For analysis of wound-induced expression, leaves were scratched with tweezers as described in Ruduś et al. (2014) and the adjacent regions were observed after 150 min. For analysis of dark-induced expression, plants were moved to a dark chamber and leaves were observed after 2 hours. Fluorescence intensities were quantified with the Leica Application Suite Advanced Fluorescence (LAS AF) software according to the manufacturer. Mean gray values of GFP fluorescence were calculated in each of three randomly selected ROI (1,000 μm^2).

3. Results and Discussion

3.1. Gateway binary vectors with an organelle-targeted fluorescent protein as a reporter

We developed Gateway binary vector series carrying an organelle-targeted sGFP or TagRFP for promoter:reporter analysis in plants. The sequences fused to fluorescent proteins for organelle localization were the signal peptide of *A. thaliana* endo-xyloglucan transferase and HDEL retention signal for ER-targeted sGFP (ER-sGFP), PKKKRKV for nucleus-targeted sGFP (NLS-sGFP) and TagRFP (NLS-TagRFP), SKL for peroxisome-targeted sGFP (Px-sGFP) and TagRFP (Px-TagRFP) and first to 57th amino acid residues including presequence of *A. thaliana* F_1ATPase γ subunit for mitochondria-targeted sGFP (Mt-sGFP) and TagRFP (Mt-TagRFP). Fig. 1 shows the schematic structure of the 56 vectors constructed in this study. The pGWB series contain *att*R1-*att*R2 acceptor sites compatible with an *att*L1-promoter-*att*L2 entry clone for preparation of an *att*B1-promoter-*att*B2-

reporter construct. The R4L1pGWB series contain *att*R4-*att*L1 acceptor sites compatible with *att*L4-promoter-*att*R1 entry clone for preparation of an *att*B4-promoter-*att*B1-reporter construct. Both pGWB and R4L1pGWB were designated as 4xx, 5xx, 6xx and 7xx, where 4,5,6, and 7 represent four kinds of plant selection markers consistent with previously developed pGWBs and R4L1pGWBs (Nakagawa et al., 2007b; Nakamura et al., 2010; Tanaka et al., 2011). In this system, 4xx refers to neomycin phosphotransferase II (NPTII) conferring kanamycin resistance (Km^r), 5xx indicates hygromycin phosphotransferase (HPT) conferring hygromycin resistance (Hyg^r), 6xx refers to bialaphos resistance gene (*bar*) conferring BASTA resistance ($BASTA^r$), and 7xx indicates UDP-N-acetylglucosamine: dolichol phosphate N-acetylglucosamine-1-P transferase (GPT) conferring tunicamycin resistance (GPT^r). These marker genes were placed in reverse orientation under regulation of the nopaline synthase (nos) promoter and followed by nos terminator (Fig. 1A). The last two digits represent the reporter type of organelle-targeted fluorescent proteins; 62 for ER-sGFP, 65 for NLS-sGFP, 68 for Px-sGFP, 71 for Mt-sGFP, 85 for NLS-TagRFP, 88 for Px-TagRFP and 91 for Mt-TagRFP (Fig. 1B). The complete nucleotide sequence of pGWBs and R4L1pGWBs developed in this study appears in GenBank/EMBL/DDBJ databases under accession nos. AP018976 to AP019003 for pGWBs and AP018948 to AP018975 for R4L1pGWBs. The pGWBs and R4L1pGWBs developed in this work are available through RIKEN BRC Experimental Plant Division (https://epd.brc.riken.jp/en/).

3.2. Localization analysis of organelle-targeted TagRFP by transient expression

To confirm the intracellular localization of organelle-targeted TagRFPs constructed in this study, we performed co-localization analysis by transient expression using established localization markers NLS-sGFP (Chiu et al., 1996), Px-sGFP (Mano et al., 2002) and Mt-

sGFP (Lee et al., 2012) as references. Pro35S:NLS-sGFP, Pro35S:NLS-TagRFP, Pro35S:Px-sGFP, Pro35S:Px-TagRFP, Pro35S:Mt-sGFP, Pro35S:Mt-TagRFP constructs were prepared by LR reaction between an *att*L4-Pro35S-*att*R1 promoter entry clone and R4L1pGWB465, 485, 468, 488, 471, 491, respectively. Pairs of sGFP and TagRFP constructs carrying the same organelle-targeting signal were delivered into Japanese leek epidermal cells by particle bombardment. Confocal microscopic analysis revealed the occurrence of nuclear co-localization of NLS-sGFP and NLS-TagRFP (Fig. 2A), peroxisomal co-localization of Px-sGFP and Px-TagRFP (Fig. 2B), and mitochondrial co-localization of Mt-sGFP and Mt-TagRFP (Fig. 2C). These results clearly indicated a correct localization of newly constructed NLS-TagRFP, Px-TagRFP, and Mt-TagRFP reporters in plant cells.

3.3. Expression analysis in transgenic A. thaliana

In order to test the performance of newly constructed vectors for highly sensitive detection of gene expression, promoter:organelle-targeted-sGFP or promoter:cDNA-organelle-targeted-sGFP constructs were prepared for the transformation of *A. thaliana*. The promoter of *A. thaliana* plastidial pyruvate kinase β subunit gene (Pl-PKβ1, AT5G52920) (Baud et al., 2007; Maeo et al., 2009) was used for the construction of ProPl-PKβ1:Px-sGFP by an LR reaction between *att*L1-ProPl-PKβ1-*att*L2 entry clone (Maeo et al., 2009) and pGWB468. The promoter and coding region of the gene for *A. thaliana* MYB21 (AT3G27810) were used for the construction of ProMYB21:MYB21-NLS-sGFP and ProMYB21:MYB21-Px-sGFP by LR reactions between *att*L1-ProMYB21:MYB21-*att*L2 entry clone and pGWB565, or pGWB468, respectively. The promoter of the gene for *A. thaliana* DAD1-LIKE LIPASE2 (DALL2, AT1G51440) was used for the construction of ProDALL2:NLS-sGFP by an LR reaction between *att*L4-ProDALL2-*att*R1 entry clone and R4L1pGWB565. The promoter of the gene for *A. thaliana* 3-ketoacyl-CoA thiolase 2 (KAT2,

AT2G33150) was used for the construction of ProKAT2:NLS-sGFP by an LR reaction between *att*L4-ProKAT2-*att*R1 entry clone and R4L1pGWB565. We also prepared ProPl-PKβ1:sGFP, ProMYB21:MYB21-sGFP, ProDALL2:sGFP and ProKAT2:sGFP expressing sGFP without any organelle-targeting signals as reference constructs by LR reactions with corresponding entry clones and pGWB404 or R4L1pGWB504. These constructs were introduced into *A. thaliana* and selected transgenic *A. thaliana* were used for confocal microscopic analysis.

The plastidial pyruvate kinase catalyzes the transphosphorylation of phosphoeneolpyruvate and ADP to pyruvate and ATP (Valentini et al., 2000), and controlling the supply of pyruvate and ATP for fatty acid synthesis in the plastids. In *A. thaliana*, Pl-PKβ1, a subunit of plastidial pyruvate kinase was shown to be expressed in the flower by promoter:GUS analysis (Baud et al., 2007). In this study, we analyzed promoter activity by ProPl-PKβ1:sGFP and ProPl-PKβ1:Px-sGFP constructs. Because the *att*L1-ProPl-PKβ1-*att*L2 entry clone carries -300 to +6 (A of initiation codon is +1) region, translation initiated from the entry clone in the binary constructs and the 13 amino acids MAHPAFLYKWDNS (third to thirteenth amino acids are derived from *att*B2 site) was added to the N-terminal of sGFP and Px-sGFP. In ProPl-PKβ1:sGFP transgenic *A. thaliana*, a faint GFP fluorescence was observed only in stigma (Fig. 3A-I). In contrast, bright peroxisome-localized GFP fluorescence was observed in ovary, stigma, style, petal, sepal, pedicel, stamen, and maturing seeds of ProPl-PKβ1:Px-sGFP transgenic *A. thaliana* (Fig. 3K-P, R). The results obtained by Px-sGFP were consistent with previous reports using promoter:GUS (Baud et al., 2007; Maeo et al., 2009). We also found weak expression in leaf and root (Fig. 3J and Q). In addition, we performed quantitative analysis and found a significant increase of fluorescence intensities in ProPl-PKβ1:Px-sGFP compared to ProPl-PKβ1:sGFP for all organs examined (Fig. 5A).

297 These results indicated that promoter activity was monitored by Px-sGFP more sensitively
298 than sGFP by accumulating expressed fluorescent protein in peroxisomes.

299 The *A. thaliana* MYB21 encodes an R2R3 MYB transcription factor controlling the
300 development of petal, stamen, and carpel by stimuli of jasmonic acid. In a histochemical
301 expression analysis of MYB21, promoter and entire coding region was used for a GUS fusion
302 construct (ProMYB21:MYB21-GUS) and GUS activity was observed in filaments, style, and
303 the vascular system of sepals and petals (Reeves et al., 2012). In this study, we analyzed
304 expression of MYB21 by ProMYB21:MYB21-sGFP, ProMYB21:MYB21-Px-sGFP and
305 ProMYB:MYB21-NLS-sGFP constructs. We observed little or no GFP fluorescence in all
306 examined organs of ProMYB21:MYB21-sGFP transgenic *A. thaliana* (Fig. 4A-F). In
307 ProMYB21:MYB21-Px-sGFP transgenic *A. thaliana*, GFP fluorescence was clearly observed
308 in the ovary and the vascular system of petals and sepals (Fig. 4H, I, J) as reported in Reeves
309 et al. (2012). We also observed strong expression in leaf, pedicel, and root (Fig. 4G, K, L).
310 The GFP signals detected in these organs showed typical peroxisome-localization pattern as
311 observed in Fig. 2B and 3, indicating that MYB21-Px-sGFP fusion protein was correctly
312 accumulated in peroxisomes. In the ProMYB21:MYB21-NLS-sGFP transgenic *A. thaliana*,
313 nucleus-localized GFP signal was observed in leaf, ovary, the vascular system of sepals and
314 petals, pedicel, and root (Fig. 4M-R). The fluorescence intensities of ProMYB21:MYB21-Px-
315 sGFP and ProMYB21:MYB21-NLS-sGFP were significantly increased compared to that of
316 ProMYB21:MYB21-sGFP in all observed organs (Fig. 5B). These results showed the
317 advantage of both Px-sGFP and NLS-sGFP for the detection of promoter activity and
318 indicated the availability of entry clones including promoter and coding regions for highly
319 sensitive expression analysis using fusion with Px-sGFP or NLS-sGFP.

320 Next, we examined expression by inducible promoters under non-induced conditions.
321 *A. thaliana* DALL2 is a homolog of DAD1 with an expression known to be induced by

322 wounding (Ruduś et al., 2014). We prepared ProDALL2:sGFP and ProDALL2:NLS-sGFP
323 using an *att*L4-ProDALL2-*att*R1 entry clone with no initiation codon and analyzed their
324 expressions in *A. thaliana*. In the leaves of ProDALL2:sGFP transgenic *A. thaliana*, we
325 observed almost no GFP signals under non-induced conditions (Fig. 6A), and found only
326 faint GFP fluorescence after wounding (Fig. 6B and I). On the other hand, nucleus-localized
327 GFP signals were detected in leaves of ProDALL2:NLS-sGFP transgenic *A. thaliana* even
328 under non-induced conditions (Fig. 6C), with clear increasing of the frequency and intensity
329 of GFP fluorescence after wounding (Fig. 6D, I). We also tested the promoter of *A. thaliana*
330 KAT2, one of 3-keto-acyl-CoA thiolase. KAT2 catalyzes the final step of β-oxidation in
331 peroxisomes for fatty acid metabolism and jasmonic acid production and its expression was
332 found to be increased by dark-induced senescence in leaves (Castillo and Leon, 2008;
333 Castillo et al., 2004). We used the *att*L4-ProKAT2-*att*R1 entry clone carrying –2074 to +6
334 region for preparation of ProKAT2:sGFP and ProKAT2:NLS-sGFP constructs. Therefore,
335 sGFP and NLS-sGFP having the additional 12 amino acids METSLYKKAGSS (third to
336 twelfth amino acids are derived from the *att*B1 site) at the N-terminal were translated in this
337 experiment. In leaves of ProKAT2:sGFP transgenic *A. thaliana*, we observed almost no GFP
338 fluorescence under non-induced conditions (Fig. 6E) and a weak GFP fluorescence after dark
339 treatment (Fig. 6F, J). In contrast, clear nucleus-localized GFP fluorescence was observed in
340 leaves of ProKAT2:NLS-sGFP transgenic *A. thaliana* even under non-induced conditions
341 (Fig. 6G) and the intensity and frequency of nucleus-localized GFP signals were drastically
342 increased after dark treatment (Fig. 6H, J). These results indicated that visualization of a
343 weak promoter activity under non-induced conditions was possible by organelle-targeted
344 GFP equipped in the vector system described here. The organelle-targeted GFP also
345 enhanced the intensity of fluorescence signals under induced conditions and made possible
346 the clear observation of gene expression.

4. Conclusions

In this study, we reported the construction and validity of the Gateway cloning technology-compatible binary vectors equipped with organelle-targeted fluorescent proteins for promoter assay in plants. Several localization targets are available in this system, including ER (ER-sGFP), nucleus (NLS-sGFP and NLS-TagRFP), peroxisome (Px-sGFP and Px-TagRFP), and mitochondria (Mt-sGFP and Mt-TagRFP). The binary vectors developed here are consisting of four selection-marker series (Km^r, Hyg^r, $BASTA^r$, and $Tunica^r$) to match a wide range of plant transformation experiments. Promoter entry clones of *att*L1-promoter-*att*L2 and *att*L4-promoter-*att*R1 types are available for preparation of promoter:organelle-targeted fluorescent protein constructs by LR reaction with developed pGWBs (*att*R1-*att*R2 acceptor sites) and R4L1pGWBs (*att*R4-*att*L1 acceptor sites), respectively. We detected brighter fluorescence by the promoter:Px-sGFP and promoter:NLS-sGFP constructs than by the promoter:sGFP construct in transgenic *A. thaliana*. The vector system developed here has the advantage of high sensitivity in promoter:reporter assays by accumulating fluorescent proteins in target organelles.

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Figure Captions

Fig. 1. Schematic illustration of the pGWBs and R4L1pGWBs. (A) The overall structure of pGWBs and R4L1pGWBs. *NPTII* (Km^r), *HPT* (Hyg^r), *bar* ($BASTA^r$), and *GPT* ($Tunica^r$) are used as plant selection markers for pGWB4xx, pGWB5xx, pGWB6xx and pGWB7xx series, respectively. pGWB and R4L1pGWB vectors contain *att*R1-*att*R2 and *att*R4-*att*L1 acceptor sites, respectively. (B) Organelle-targeted fluorescent proteins used as tags in pGWBs and R4L1pGWBs. ER-sGFP, ER-targeted sGFP; NLS-sGFP, nucleus-targeted sGFP; Px-sGFP, peroxisome-targeted sGFP; Mt-sGFP, mitochondria-targeted sGFP; NLS-TagRFP, nucleus-targeted TagRFP; Px-TagRFP, peroxisome-targeted TagRFP; Mt-TagRFP, mitochondria-targeted TagRFP; RB, right border; LB, left border; Cm^r, chloramphenicol resistance (chloramphenicol acetyl transferase) used for selection in bacteria; *ccd*B, negative selection marker used in bacteria; sGFP, synthetic green fluorescent protein with S65T mutation; TagRFP, Tag red fluorescent protein; *sta*, region for stability in *Agrobacterium tumefaciens*; *rep*, broad host-range replication origin; *bom*, cis-acting element for conjugational transfer; *ori*, ColE1 replication origin; *aad*A, spectinomycin resistance (Spc^r) marker used for selection in bacteria.

Fig. 2. Intracellular localization of organelle-targeted sGFP and TagRFP transiently expressed in Japanese leek epidermal cells. (A) Pro35S:NLS-sGFP + Pro35S:NLS-TagRFP. (B) Pro35S:Px-sGFP + Pro35S:Px-TagRFP. (C) Pro35S:Mt-sGFP + Pro35S:Mt-TagRFP. GFP, signal of sGFP; RFP, signal of TagRFP; Overlay, overlay of GFP and RFP. Scale bars =10 μm.

Fig. 3. Expression of sGFP and Px-sGFP driven by Pl-PKβ1 promoter in transgenic *A. thaliana*. (A-I) images of ProPl-PKβ1:sGFP. (J-R) images of ProPl-PKβ1:Px-sGFP. (A and J) leaf, (B and K) ovary, (C and L) stigma + style, (D and M) petal, (E and N) sepal, (F and O) pedicel, (G and P) stamen, (H and Q) root, (I and R) seed. AF, autofluorescence; Overlay, overlay of GFP, AF, and differential interference contrast (DIC). Scale bars =10μm.

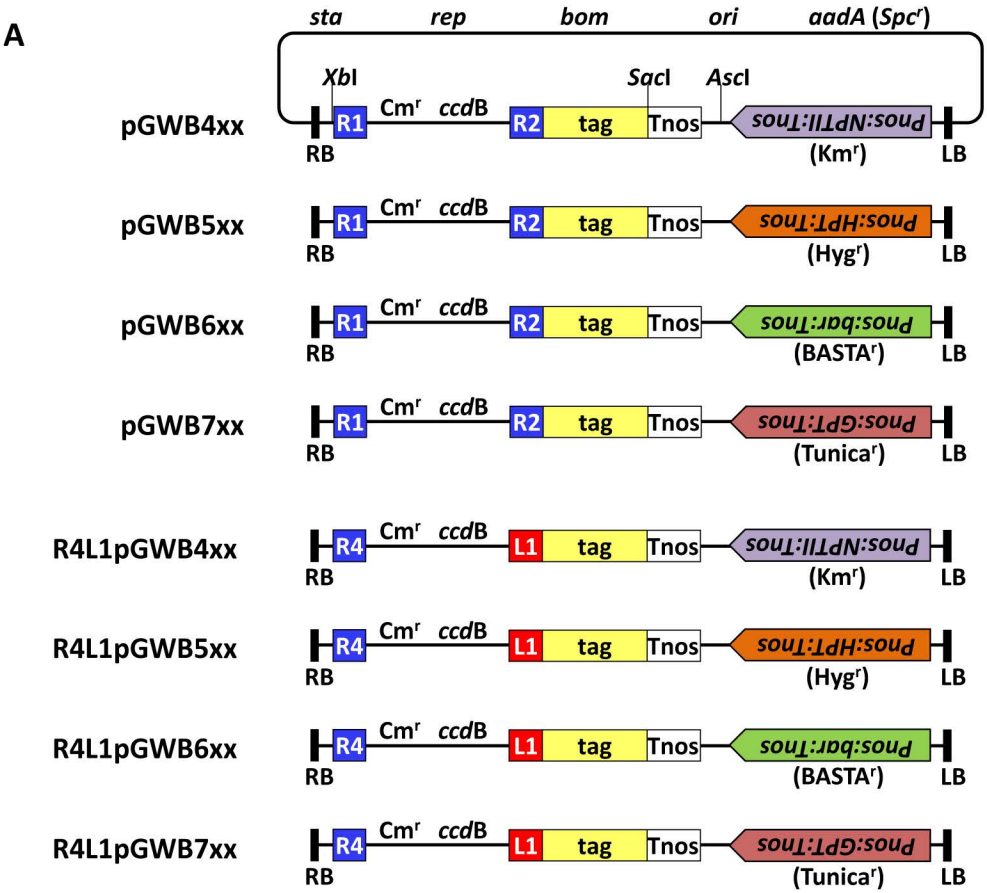
Fig. 4. Expression of MYB21-sGFP, MYB21-Px-sGFP and MYB21-NLS-sGFP driven by MYB21 promoter in transgenic *A. thalian*a. (A-F) images of ProMYB21:MYB21-sGFP. (G-L) images of ProMYB21:MYB21-Px-sGFP. (M-R) images of ProMYB21:MYB21-NLS-sGFP. (A, G, M) leaf, (B, H, N) ovary, (C, I, O) petal, (D, J, P) sepal, (E, K, Q) pedicel, (F, L, R) root. AF, autofluorescence; Overlay, overlay of GFP, AF, and DIC. Scale bars = 10μm.

Fig. 5. Comparison of fluorescence intensities of sGFP and organelle-targeted sGFP among organs in transgenic *A. thaliana*. (A) Fluorescence intensities of ProPl-PKβ1:sGFP and ProPl-PKβ1:Px-sGFP in various organs. (B) Fluorescence intensities of ProMYB21:MYB21-sGFP, ProMYB21:MYB21-Px-sGFP and ProMYB21:MYB21-NLS-sGFP in various organs. Error bars represent SD. **p*<0.01, Student's *t*-test.

Fig. 6. Expression and comparison of fluorescence intensities of sGFP and NLS-sGFP driven by DALL2 and KAT2 promoters in transgenic *A. thaliana*. (A and B) images of ProDALL2:sGFP. (C and D) images of ProDALL2:NLS-sGFP. (A and C) non-induced condition, (B and D) 150 min after wounding. (E and F) images of ProKAT2:sGFP. (G and H) images of ProKAT2:NLS-sGFP. (E and G) non-induced condition, (F and H) 2 hr after

dark. (I) Fluorescence intensities in leaves of ProDALL2:sGFP and ProDALL2:NLS-sGFP after wound treatment. (J) Fluorescence intensities in leaves of ProKAT2:sGFP and ProKAT2:NLS-sGFP after dark treatment. AF, autofluorescence; Overlay, overlay of GFP, AF, and DIC. Scale bars = 10μm. Error bars represent SD. *p<0.01, Student's t-test.

A



B

ER-sGFP	NLS-sGFP	Px-sGFP	Mt-sGFP
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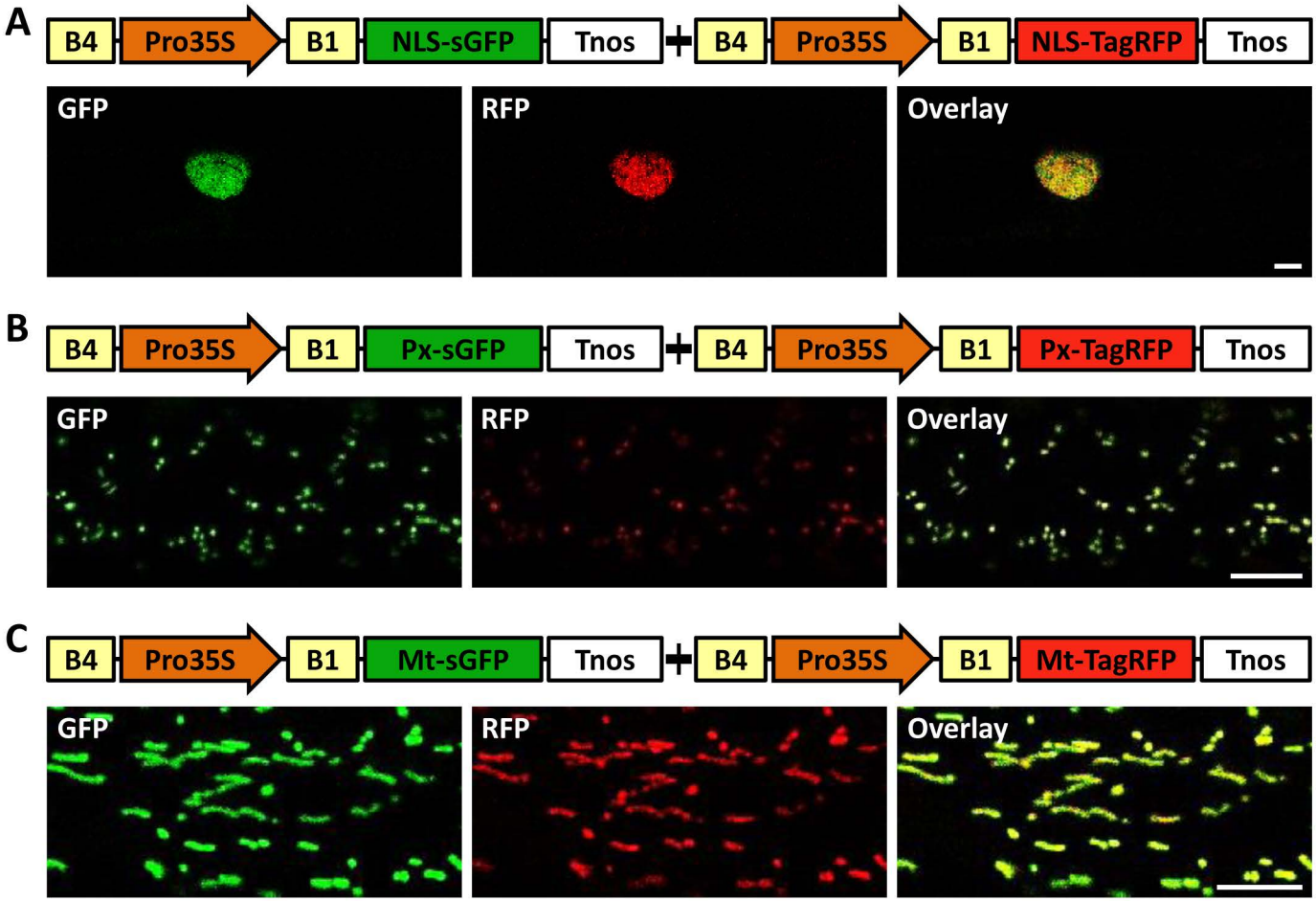
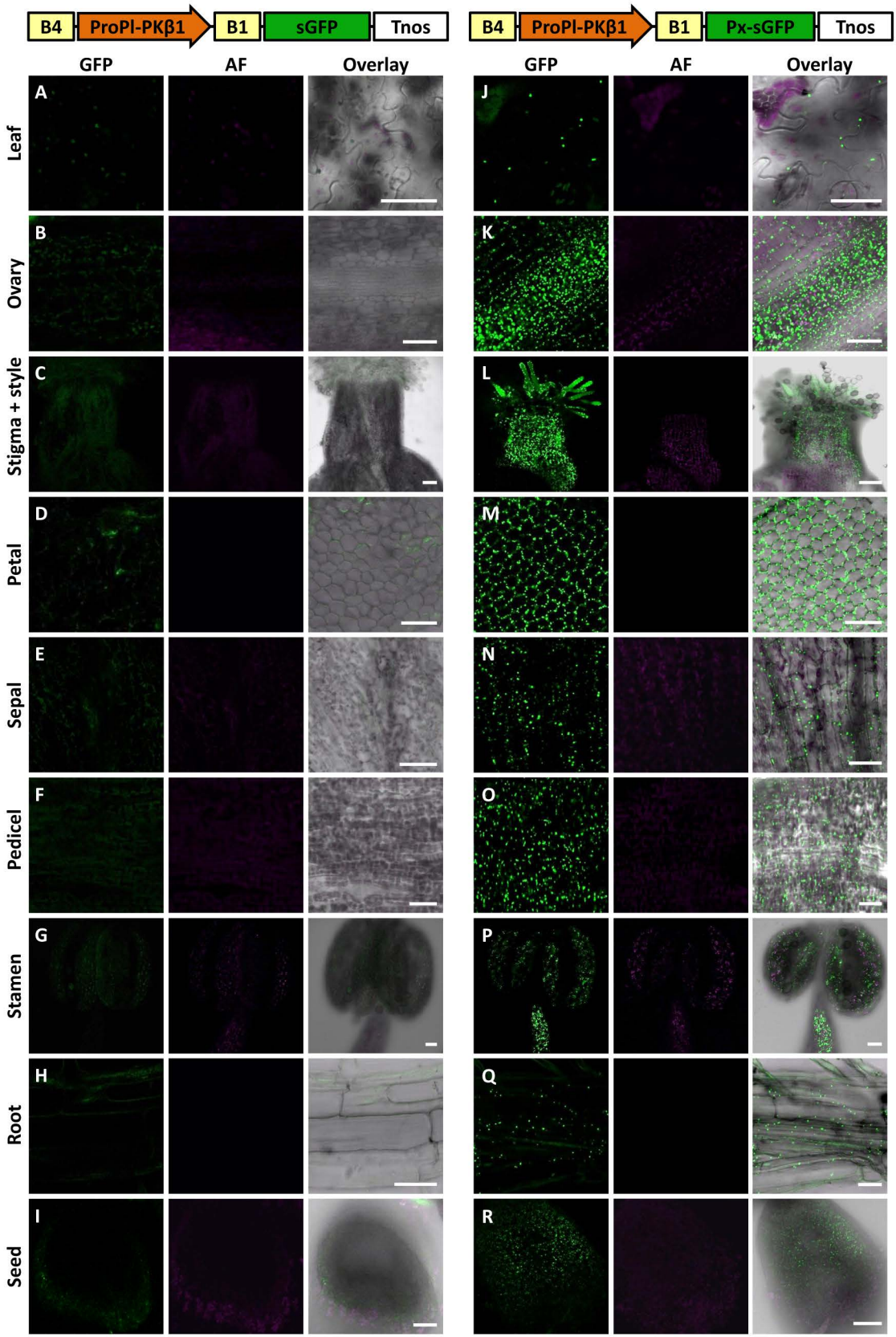
pGWB562	pGWB565	pGWB568	pGWB571
pGWB662	pGWB665	pGWB668	pGWB671
pGWB762	pGWB765	pGWB768	pGWB771
R4L1pGWB462	R4L1pGWB465	R4L1pGWB468	R4L1pGWB471
R4L1pGWB562	R4L1pGWB565	R4L1pGWB568	R4L1pGWB571
R4L1pGWB662	R4L1pGWB665	R4L1pGWB668	R4L1pGWB671
R4L1pGWB762	R4L1pGWB765	R4L1pGWB768	R4L1pGWB771

NLS-TagRFP	Px-TagRFP	Mt-TagRFP
pGWB485	pGWB488	pGWB491
pGWB585	pGWB588	pGWB591
pGWB685	pGWB688	pGWB691
pGWB785	pGWB788	pGWB791
R4L1pGWB485	R4L1pGWB488	R4L1pGWB491
R4L1pGWB585	R4L1pGWB588	R4L1pGWB591
R4L1pGWB685	R4L1pGWB688	R4L1pGWB691
R4L1pGWB785	R4L1pGWB788	R4L1pGWB791


A
B4
Pro35S
B1
NLS-sGFP
Tnos
B4
Pro35S
B1
NLS-TagRFP
Tnos
GFP
RFP
Overlay
B
B4
Pro35S
B1
Px-sGFP
Tnos
B4
Pro35S
B1
Px-TagRFP
Tnos
GFP
RFP
Overlay
C
B4
Pro35S
B1
Mt-sGFP
Tnos
B4
Pro35S
B1
Mt-TagRFP
Tnos
GFP
RFP
Overlay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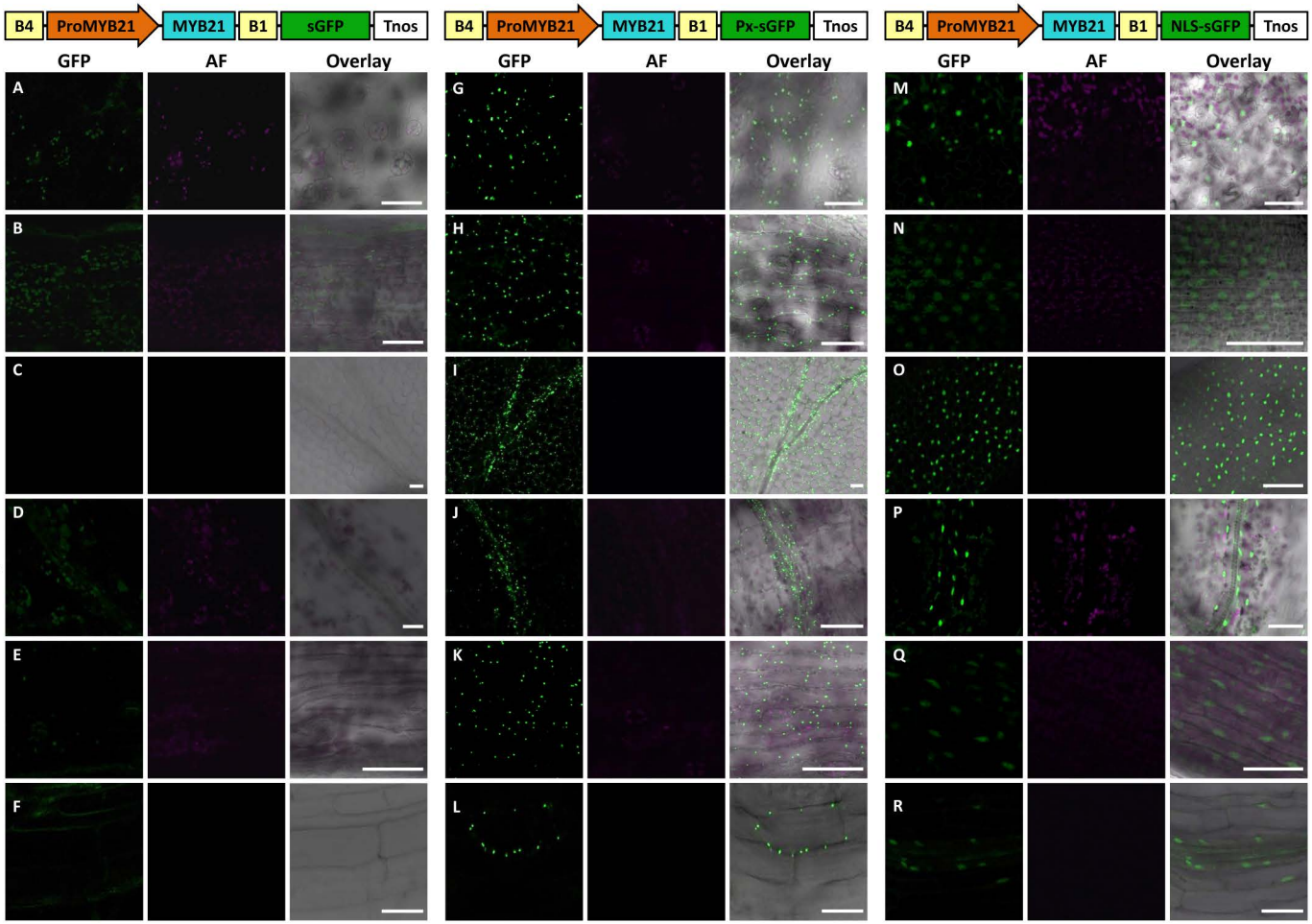
ProPl-PKβ1
B1
sGFP
Tnos
B4
ProPl-PKβ1
B1
Px-sGFP
Tnos
GFP
AF
Overlay
GFP
AF
Overlay
Leaf
Ovary
Stigma + style
Petal
Sepal
Pedicel
Stamen
Root
Seed
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R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ProMYB21
MYB21
B1
sGFP
Tnos
B4
ProMYB21
MYB21
B1
Px-sGFP
Tnos
B4
ProMYB21
MYB21
B1
NLS-sGFP
Tnos
GFP
AF
Overlay
GFP
AF
Overlay
GFP
AF
Overlay
Leaf
Ovary
Petal
Sepal
Pedicel
Root
A
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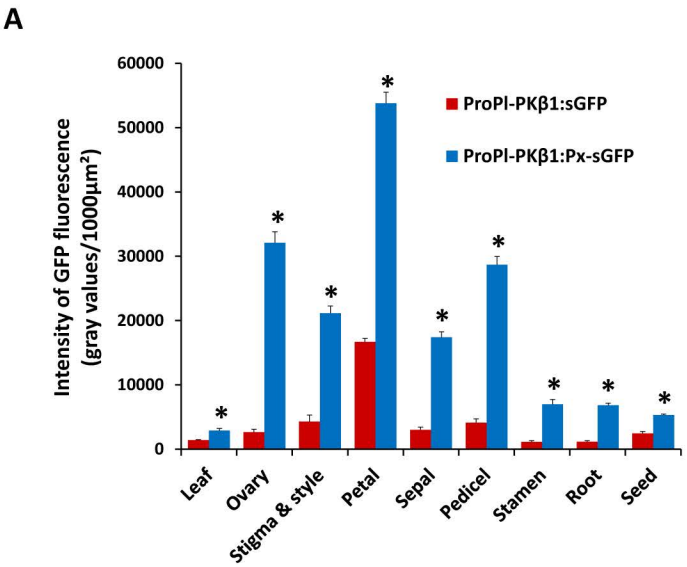
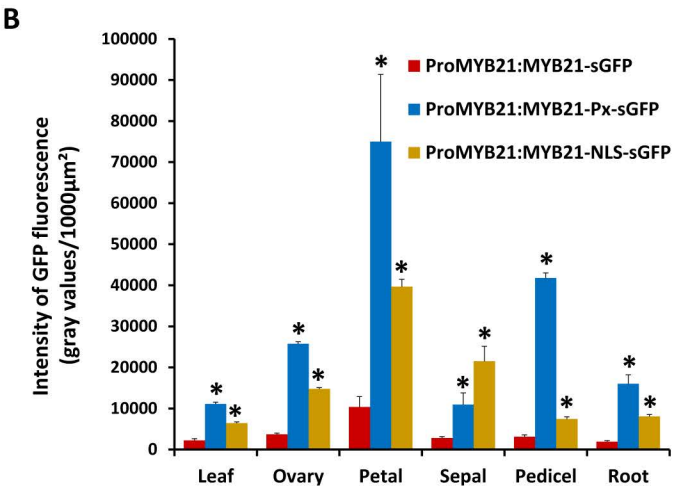

A
Intensity of GFP fluorescence (gray values/1000μm²)
60000
50000
40000
30000
20000
10000
0
ProPl-PKβ1:sGFP
ProPl-PKβ1:Px-sGFP
Leaf
Ovary
Stigma & style
Petal
Sepal
Pedicel
Stamen
Root
Seed


B
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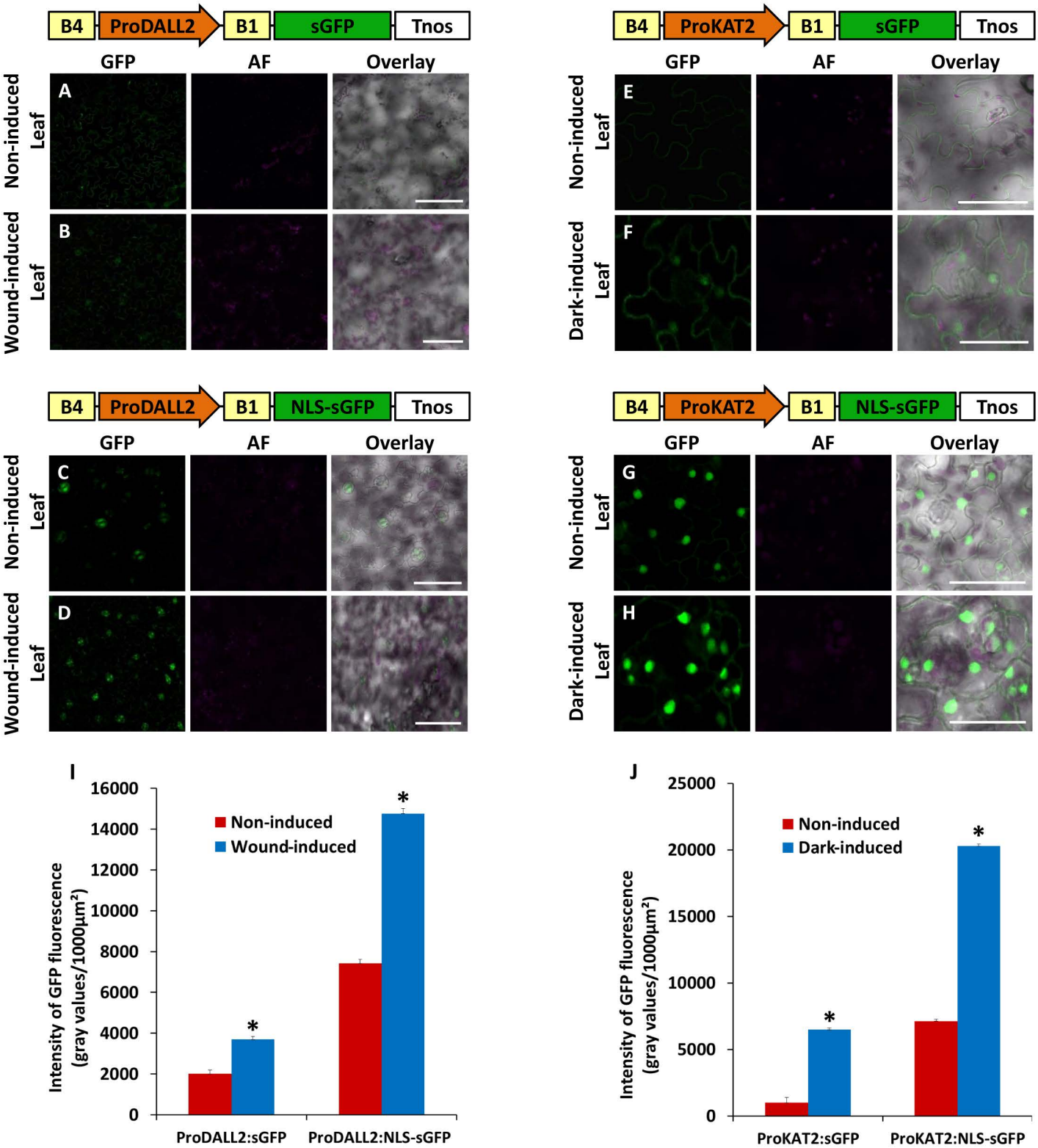
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
ProMYB21:MYB21-sGFP
ProMYB21:MYB21-Px-sGFP
ProMYB21:MYB21-NLS-sGFP
Leaf
Ovary
Petal
Sepal
Pedicel
Root


B4
ProDALL2
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sGFP
Tnos
GFP
AF
Overlay
Non-induced Leaf
Wound-induced Leaf
A
B
B4
ProKAT2
B1
sGFP
Tnos
GFP
AF
Overlay
Non-induced Leaf
Dark-induced Leaf
E
F
B4
ProDALL2
B1
NLS-sGFP
Tnos
GFP
AF
Overlay
Non-induced Leaf
Wound-induced Leaf
C
D
B4
ProKAT2
B1
NLS-sGFP
Tnos
GFP
AF
Overlay
Non-induced Leaf
Dark-induced Leaf
G
H
I
Intensity of GFP fluorescence (gray values/1000μm²)
16000
14000
12000
10000
8000
6000
4000
2000
0
Non-induced
Wound-induced
*
*
ProDALL2:sGFP
ProDALL2:NLS-sGFP
J
Intensity of GFP fluorescence (gray values/1000μm²)
25000
20000
15000
10000
5000
0
Non-induced
Dark-induced
*
*
ProKAT2:sGFP
ProKAT2:NLS-sGFP