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TECHNIQUES FOR MOLECULAR ANALYSIS

Gateway-compatible vectors for plant functional genomics and proteomics

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Summary

Gateway cloning technology facilitates high-throughput cloning of target sequences by making use of the bacteriophage lambda site-specific recombination system. Target sequences are first captured in a commercially available 'entry vector' and are then recombined into various 'destination vectors' for expression in different experimental organisms. Gateway technology has been embraced by a number of plant laboratories that have engineered destination vectors for promoter specificity analyses, protein localization studies, protein/protein interaction studies, constitutive or inducible protein expression studies, gene knockdown by RNA interference, or affinity purification experiments. We review the various types of Gateway destination vectors that are currently available to the plant research community and provide links and references to enable additional information to be obtained concerning these vectors. We also describe a set of 'pEarleyGate' plasmid vectors for *Agrobacterium*-mediated plant transformation that translationally fuse FLAG, HA, cMyc, AcV5 or tandem affinity purification epitope tags onto target proteins, with or without an adjacent fluorescent protein. The oligopeptide epitope tags allow the affinity purification, immunolocalization or immunoprecipitation of recombinant proteins expressed *in vivo*. We demonstrate the utility of pEarleyGate destination vectors for the expression of epitope-tagged proteins that can be affinity captured or localized by immunofluorescence microscopy. Antibodies detecting the FLAG, HA, cMyc and AcV5 tags show relatively little cross-reaction with endogenous proteins in a variety of monocotyledonous and dicotyledonous plants, suggesting broad utility for the tags and vectors.

Keywords: affinity purification, epitope tag, fusion protein, protein localization, recombinational cloning.

Introduction

Moving beyond gene discovery to understanding gene function is facilitated by the ability to easily express proteins from cloned genes in both homologous and non-homologous biological contexts. For instance, expression in plants of a protein engineered to include an oligopeptide epitope tag can allow affinity purification or immunoprecipitation of that protein and any associated proteins (Fritze and Anderson, 2000; Jarvik and Telmer, 1998). This can be an extremely useful approach for the isolation, identification and biochemical analysis of multi-protein complexes. Similarly, fusing an open reading frame to a fluorescent protein, such as green, yellow, red or cyan fluorescent proteins (GFP, YFP, RFP or CFP, respectively), can be useful for determining the subcellular localization of a protein and for testing for interactions with other fluorescently tagged proteins within living cells (Ehrhardt, 2003; Hanson and Kohler, 2001; Haseloff, 1999; Stewart, 2001). A researcher might also find it useful to express a target protein in *Escherichia coli* or insect cells in order to test for enzymatic activities, to produce sufficient recombinant protein for raising antibodies, or to perform protein interaction studies. Engineering multiple expression vector constructs to accomplish these goals for every target gene of interest using traditional ligase-mediated cloning is time-consuming and laborious, posing a technical barrier for high-throughput functional genomics or proteomics projects. Fortunately, such barriers have been

lowered considerably by the advent of Gateway cloning technology (Hartley *et al.*, 2000).

Gateway cloning exploits the bacteriophage lambda recombination system, thereby bypassing the need for traditional ligase-mediated cloning. Once captured in a Gateway-compatible plasmid 'entry vector', an open reading frame or gene flanked by recombination sites can be recombined into a variety of 'destination vectors' that possess compatible recombination sites. Destination vectors for protein expression in *E. coli*, yeast, mammalian, and insect cells are commercially available and are marketed by Invitrogen (Carlsbad, CA, USA). Although Gateway-compatible plant destination vectors for expression of proteins in transgenic plants are not commercially available at the present time, a number of laboratories have engineered such vectors (Table 1; Figure 1). These plant destination vectors have been designed for a variety of specific purposes including protein localization, promoter functional analysis, gene overexpression, gene knockdown by RNA interference, production of epitope-tagged proteins for affinity purification, or analysis of protein/protein interactions using fluorescence resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET) or bimolecular fluorescence complementation (BiFC).

In addition to reviewing previously described Gateway-compatible plant destination vectors, we describe a series of pEarleyGate vectors that we designed for transient or stable expression of proteins fused to a variety of oligopeptide epitope tags and/or GFP, YFP or CFP. Representative immunoblotting, affinity purification and protein localization data are provided in order to illustrate the usefulness of pEarleyGate vectors.

Gateway cloning

The Gateway cloning system exploits the accurate, site-specific recombination system utilized by bacteriophage lambda in order to shuttle sequences between plasmids bearing compatible recombination sites (Figure 2). In the Pikaard laboratory, the preferred method for initially capturing sequences of interest is to use topoisomerase-mediated cloning (Shuman, 1994), which eliminates the

Table 1 Gateway compatible plant destination vectors

References	Uses for vectors	Reporter genes/tags	Website
Karimi *et al.* (2002)	Promoter analysis Inducible expression Protein localization RNAi	GUS, GFP, YFP, CFP, Luciferase	http://www.psb.ugent.be/gateway/
Helliwell and Waterhouse (2003)	RNAi		http://www.pi.csiro.au/rnai/hithroughput.htm
Curtis and Grossniklaus (2003)	Promoter analysis Inducible expression Protein localization RNAi	GFP, GUS, His	http://www.unizh.ch/botinst/devo_website/curtisvector/
Joubes *et al.* (2004)	Inducible expression		http://www.psb.ugent.be/gateway/
Bensmihen *et al.* (2004)	Epitope tagging Activation domain addition	HA, VP16	http://www.isv.cnrs-gif.fr/jg/alligator/vectors.html
Rohila *et al.* (2004)	TAP protein purification	Protein A IgG binding domain, calmodulin	
Walter *et al.* (2004)	BiFC	Truncated C- and N-termini of YFP for BiFC	
Lo *et al.* (2005)	Inducible RNAi		
Rubio *et al.* (2005)	TAP protein purification	Protein A IgG binding domain, cMyc-His	
Tzfira *et al.* (2005)	Protein localization	GFP	
Karimi *et al.* (2005)	Multicomponent recombination		http://www.psb.ugent.be/gateway/
Albrecht von Arnim (University of Tennessee, Knoxville, TN, USA, personal communication)	BRET	Luciferase, YFP	http://www.bio.utk.edu/vonarnim/BRET/BRET-vectors.html
This article	Protein localization Affinity purification Immunolocalization	HA, FLAG, cMyc, AcV5, TAP, His, GFP, YFP, CFP	http://www.biology.wustl.edu/pikaard/pearleygate%20plasmid%20vectors/pearleygate%20homepage.html

BiFC, bimolecular fluorescence complementation; BRET, bioluminescence resonance energy transfer; GFP, YFP and CFP, green, yellow and cyan fluorescent proteins, respectively; RNAi, RNA interference; TAP, tandem affinity purification; His, histidine; HA, cMyc, FLAG and AcV5 are epitope tags (see page 11 for sequences).

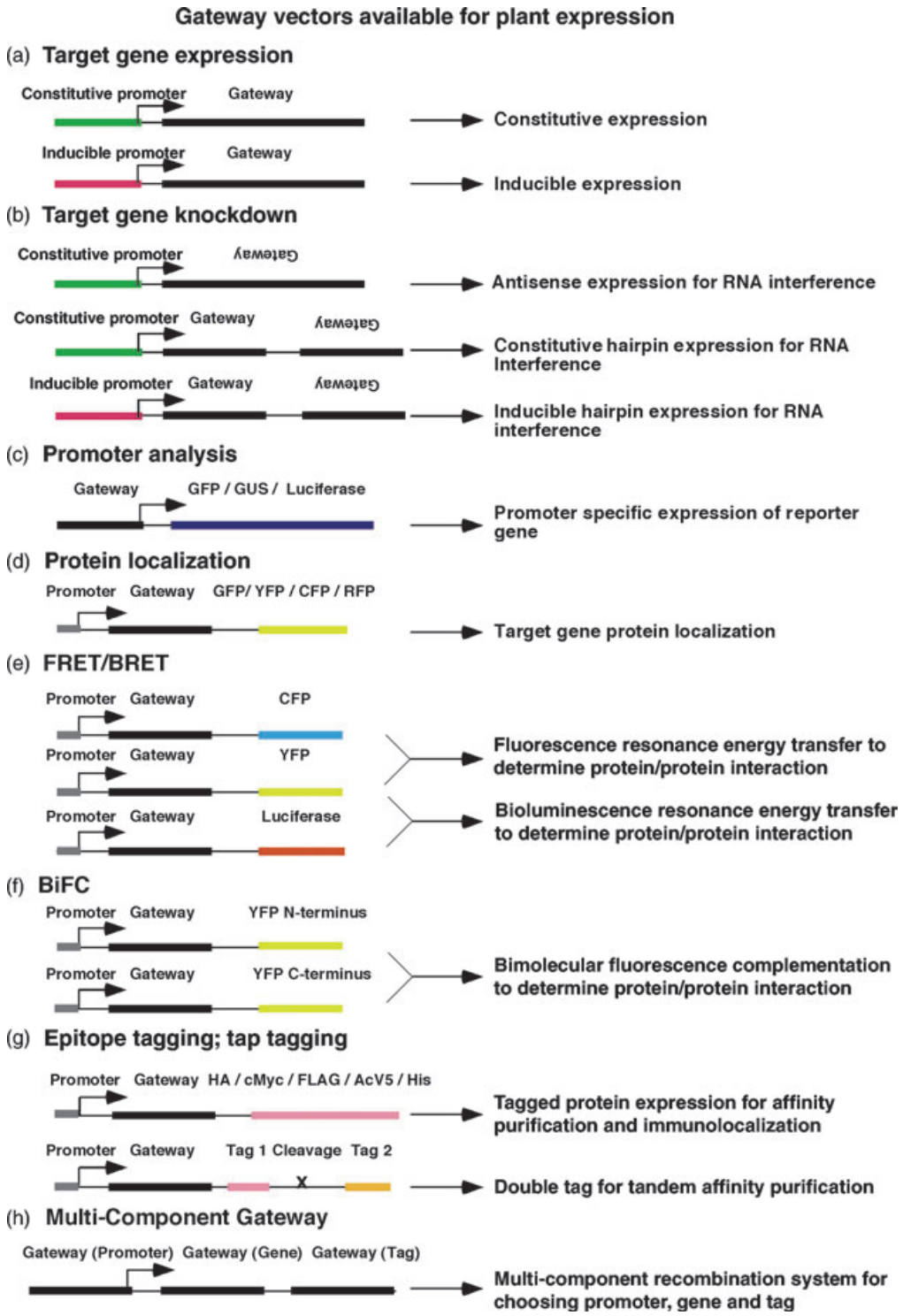
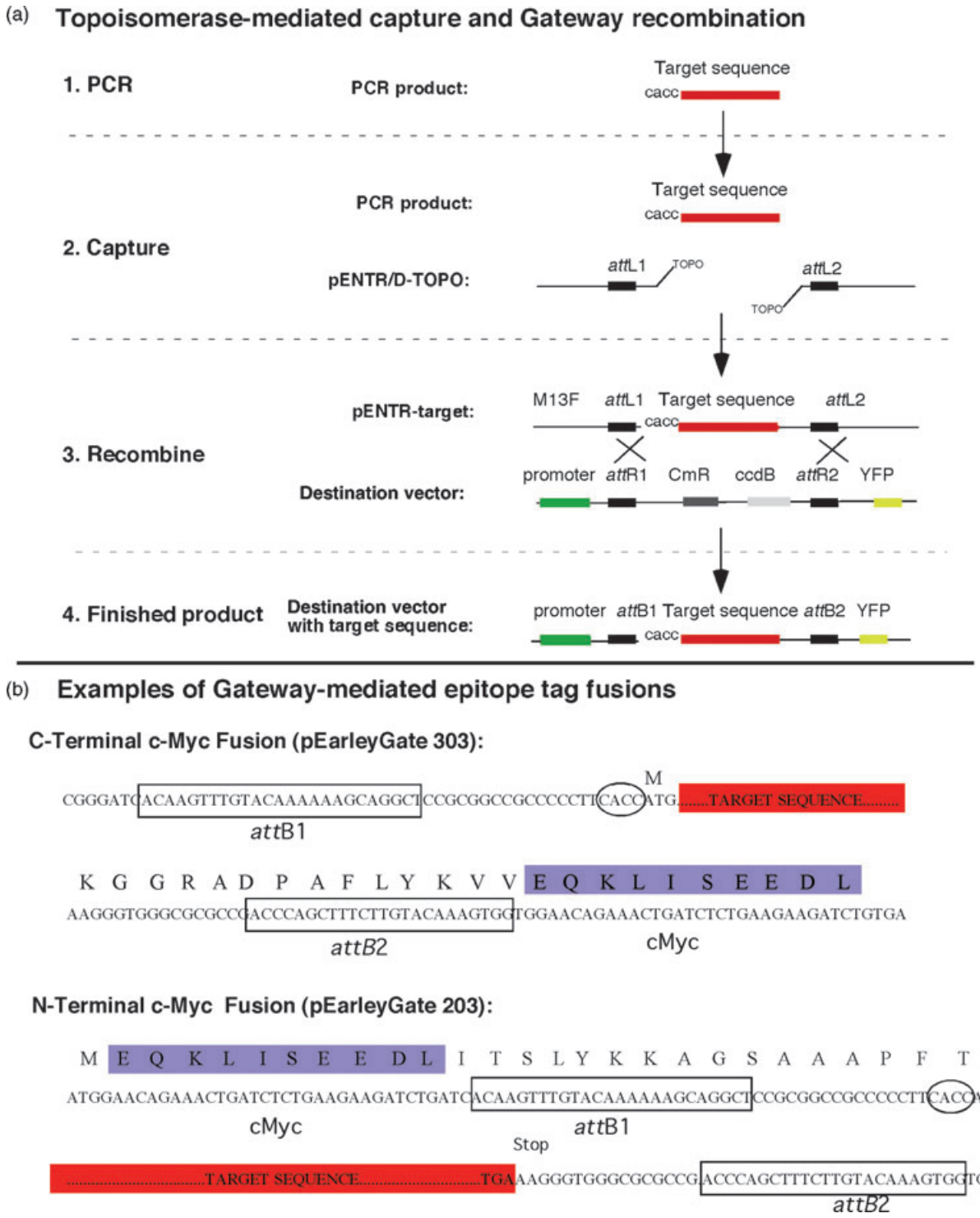


Figure 1. A summary of available Gateway-compatible vectors for use in plants.
Diagrams illustrate Gateway-compatible vectors for (a) protein overexpression, (b) RNA knockdown, (c) promoter analysis, (d) protein subcellular localization, (e) fluorescence resonance energy transfer and bioluminescence resonance energy transfer, (f) bimolecular fluorescence complementation, (g) epitope tagging and tandem affinity purification, and (h) multi-component transgene assembly. All vectors contain *att*R recombination sites and a *ccdB* cassette for selection of successful recombination events. Only C-terminal fusions are illustrated in this figure but, for most constructs, N-terminal constructs are also available. Table 1 provides links by which more detailed information concerning available vectors can be obtained.

need for conventional DNA ligase-mediated molecular cloning. In this approach, one uses polymerase chain reaction (PCR) to amplify the target sequence using a forward primer that includes the sequence CACC at the 5′ end. This sequence facilitates directional incorporation into Invitrogen's pENTR/D-TOPO entry vector (Figure 2a, steps 1 and 2). The resulting recombinant plasmid has the target DNA sequences flanked by *att*L recombination sequences.



Once flanked by *att*L recombination sites, the sequence can be recombined with *att*R sites using the LR clonase reaction mix (Invitrogen). This reaction transfers the target sequence into a desired destination vector (Figure 2a, steps 3 and 4). Destination vectors contain a gene (*ccdB*) that is lethal to most strains of *E. coli*. 'Empty' destination vectors are therefore selected against upon transformation of *E. coli* cells with the recombination reaction. This negative selection, combined with positive selection for an antibiotic resistance marker, ensures that resulting colonies contain plasmids that have undergone recombination. The ease and speed with which a captured target sequence can be shuttled simultaneously into a variety of destination vectors are great advantages for high-throughput functional genomics/proteomics investigations.

Although we use topoisomerase-mediated cloning almost exclusively for capturing target sequences in entry vectors, there are other options. One option is to use traditional ligase-mediated insertion of a target sequence into an entry vector at a multiple cloning site that is flanked by *att*L sites. A second option is to use PCR primers that include *att*B sites when amplifying the target sequence. The resulting PCR products can be recombined directly into a donor vector containing *att*P recombination sites using the BP clonase reaction mix (Invitrogen). This BP recombination reaction results in the target sequence being flanked by *att*L sequences, which allows subsequent recombination with a destination vector. These options, as well as detailed protocols, are described in the Gateway cloning manual(s) available from Invitrogen's website (http://www.invitrogen.com).

Gateway-compatible destination vectors for use in plants

A number of laboratories have developed Gateway-compatible plant expression vectors in recent years, each designed with a specific purpose in mind (Table 1; Figure 1). Many of these plasmid vectors can replicate in both *E. coli* and *Agrobacterium tumefaciens* and possess left border and right border sequences for *Agrobacterium*-mediated T-DNA transfer. The different types of vectors, their key features and uses, URLs for websites where more information can be obtained, and pertinent references are summarized in Table 1. In some cases, the vectors can only be obtained by interested researchers though a Materials Transfer Agreement (MTA) with the laboratory and institution that engineered the plasmids. However, some vectors, including the complete set of pEarleyGate vectors, do not require an MTA and are freely available through the Arabidopsis Biological Resource Center (Columbus, OH, USA).

Plant destination vectors for constitutive or inducible gene expression

It is often useful to express a gene or open reading frame ectopically from a constitutive promoter in order to test its function in a variety of cell types. Alternatively, one might wish to control when the gene is expressed by making use of an inducible promoter. Gateway-compatible vectors have been designed for both purposes (Figure 1a). For instance, in addition to vectors that allow the expression of cloned target sequences from the strong, constitutive 35S promoter of cauliflower mosaic virus, Curtis and Grossniklaus have engineered vectors that make use of a heat-shock gene promoter or an estrogen-responsive promoter (Curtis and Grossniklaus, 2003).

An inducible Gateway-compatible expression vector that allows tighter control of gene expression than previously designed inducible systems has recently been described. This 'double-lock' inducible system requires both heat shock induction and dexamethasone-inducible control of cellular targeting of cyclization recombination (CRE) recombinase in order to activate a promoter disrupted by a DNA fragment flanked by locus of X-over P1 sites. Specifically, heat shock is used to induce the expression of CRE recombinase fused to the hormone-binding domain of the rat glucocorticoid receptor. The resulting protein remains sequestered in the cytoplasm until dexamethasone treatment, which allows the protein to move into the nucleus, catalyze the removal of the sequence blocking transcription by the 35S promoter, and thereby allow expression of the target gene (Joubes *et al.*, 2004).

Figure 2. Overview of Gateway cloning for generation of fusion proteins

(a) Topoisomerase-mediated capture and Gateway recombinational cloning of target sequences.

(1) A sequence of interest (e.g. a cDNA open reading frame) is amplified by PCR using a forward oligonucleotide primer that has the sequence CACC preceding the sequence of interest in order to facilitate direction cloning into the pENTR/D-TOPO vector (obtained from Invitrogen). A proofreading polymerase that generates PCR products with blunt ends is required. (2) PCR products are mixed with the pENTR/D-TOPO vector, which has covalently attached topoisomerase molecules that catalyze ligation of target and vector sequences. *att*L1 and *att*L2 sites flanking the cloning site mediate subsequent recombination reactions. (3) Using the LR clonase reaction enzyme mix (Invitrogen), which contains the enzymes required for recombination between *att*L and *att*R sites, the target sequence is recombined into a destination vector of choice. Located between the *att*R sites of the destination vector is a chloramphenicol resistance gene (*CmR*) and a *ccdB* gene which is lethal to most strains of *Escherichia coli*. As a result, only those *E. coli* transformed with plasmids having undergone successful recombination events survive (4).

(b) Examples of Gateway-mediated addition of cMyc epitope tags to the C-terminal or N-terminal ends of a target sequence in pEarleyGate 303 or 203, respectively. The *att*B sites (boxed) result from *att*L–*att*R recombination. The CACC sequence added at the 5′ end of the PCR-amplified target sequence is circled. Amino acids are indicated using a single-letter code. Note that additional amino acids derived from *att* sites and adjacent pENTR vector sequences are added to the translated protein.

Plant destination vectors for gene knockdown by the RNA interference (RNAi)

As first shown by Waterhouse *et al*. (Waterhouse *et al*., 1998), expression of double-stranded RNA is sufficient to trigger the RNAi pathway in plants, leading to the degradation of homologous mRNAs (Baulcombe, 2004). Production of a double-stranded RNA trigger is relatively easy to accomplish by cloning two copies of a target gene segment, in inverted orientation relative to one another, downstream of a strong promoter. Destination vectors that make use of Gateway cloning in order to capture a given trigger RNA sequence in both the forward and reverse orientations have been designed by Helliwell and Waterhouse and are named 'pHellsgate' vectors (Helliwell and Waterhouse, 2003; Wesley *et al*., 2001) (Figure 1b). Similar vectors have been designed by Karimi *et al*. (Karimi *et al*., 2002). An alternative approach is to simply produce a full-length antisense transcript to a given target cDNA by cloning the gene sequence in reverse orientation relative to the promoter (Figure 1b). If the antisense transcript anneals with the endogenous mRNA, the resulting double-stranded RNA can trigger the RNAi response. Karimi *et al*. have engineered pairs of Gateway-compatible destination vectors that allow expression of either sense or antisense transcripts of a cloned target sequence (Karimi *et al*., 2002).

Recently, an ethanol-inducible Gateway-compatible pHellsgate vector that allows reversible expression of dsRNA has been described (Lo *et al*., 2005). Because knockdown can be induced by the addition of ethanol and reversed by removal (or evaporation) of the ethanol, transcriptional gene silencing can be controlled. This system can potentially allow the conditional knockdown of essential genes for which constitutive knockdown might be lethal. Knockdown of target genes at specific times in development is also possible using this strategy.

Plant destination vectors for promoter analysis

Expression patterns for a given gene can be investigated by fusing the promoter of that gene to a reporter coding sequence and then determining the organs, cell types and developmental stages in which the reporter protein is expressed. To simplify the making of constructs for this purpose, Gateway-compatible vectors have been designed that allow promoter sequences to be recombined into plant destination vectors upstream of B-glucuronidase (GUS) or GFP reporter genes (Curtis and Grossniklaus, 2003; Karimi *et al*., 2002) (Figure 1c). GUS enzymatic activity converts a colorless substrate (X-Gluc) into a product that is an intense blue color and can be used in tissues cleared of chlorophyll and other natural pigments in order to achieve sensitive detection of transgene expression. A potential disadvantage, however, is that these methods are destructive and kill the plant cells that are analyzed. By contrast, GFP or other fluorescent proteins (e.g. YFP, CFP or RFP) can be visualized in living cells and can be monitored over time. Weakly expressed fluorescent proteins may escape detection, however, as a result in part of background fluorescence from endogenous plant pigments. By fusing GUS and GFP open reading frames, some vectors allow both reporters to be simultaneously expressed, allowing one to choose which reporter assay to employ (Karimi *et al*., 2002).

Plant destination vectors for subcellular protein localization and detection of protein/protein interactions

Unlike the vectors described above for promoter analyses, translational fusion of a protein to a fluorescent protein allows the subcellular localization of the protein to be determined. Gateway-compatible vectors that fuse GFP, YFP, CFP or RFP to either the C-terminus or the N-terminus of a target protein have been engineered by several laboratories (Curtis and Grossniklaus, 2003; Karimi *et al*., 2002; Tzfira *et al*., 2005) (Figure 1d–f). In some cases, the vectors have been designed such that a six-histidine tag (His tag) is added to the fluorescent protein (Curtis and Grossniklaus, 2003) to facilitate affinity purification of the protein on nickel-chelating resin. An alternative is provided by pEarleyGate vectors that have an influenza A virus haemagglutinin (HA) epitope tag fused to the fluorescent protein, allowing immunological affinity purification or immunoprecipitation (see description of pEarleyGate vectors below).

Gateway-compatible vectors that add YFP, CFP or luciferase to target proteins can also be useful for assaying protein/protein interactions *in vivo* using FRET, BRET or BiFC (Figure 1e,f). FRET makes use of photons emitted by CFP in order to excite YFP. Therefore, detection of YFP emission upon CFP excitation indicates a physical interaction between the proteins fused to CFP and YFP. BRET is a related phenomenon, which utilizes luciferase emissions to excite YFP. Gateway-compatible vectors for both of these applications are currently available (Karimi *et al*., 2002, Albrecht von Arnim, University of Tennessee, Knoxville, TN, USA, pers. comm.). Walter *et al*. also describe Gateway-compatible vectors that facilitate BiFC assays, in which non-fluorescent N- and C-terminal fragments of YFP must dimerize to reconstitute YFP fluorescence (Walter *et al*., 2004).

Epitope tagging vectors for protein purification

A number of groups, including ours, have created Gateway-compatible plant destination vectors that add one or more epitope tags to target proteins (Bensmihen *et al*., 2004; Rohila *et al*., 2004; Rubio *et al*., 2005) (Figure 1g). Epitope tags are short, hydrophilic peptide sequences recognized by specific antibodies. Compared with larger protein fusions, the small

size of epitope tags makes them less likely to interfere with protein folding and function (Fritze and Anderson, 2000; Jarvik and Telmer, 1998). Epitope tags recognized by monoclonal or monospecific antibodies offer a means of efficient detection, affinity purification, or subcellular localization of tagged proteins. Expression of recombinant proteins bearing epitope tags can also eliminate the need to generate antibodies recognizing each new protein to be studied, which can be problematic as a result of low antigenicity or high background cross-reaction with other proteins. Single epitope or tandem affinity peptide (TAP) tags are increasingly used to facilitate large-scale, high-throughput proteomics studies (Gavin *et al.*, 2002; Ho *et al.*, 2002). Two groups have recently described Gateway-compatible TAP tagging vectors for use in plants. Rohila *et al.* described a TAP tag containing two copies of the immunoglobulin G (IgG) binding domain of *Staphylococcus aureus* protein A separated from a calmodulin-binding peptide by an intervening Tobacco Etch Virus (TEV) cleavage site (Rohila *et al.*, 2004). Rubio *et al.* described a TAP tag containing two IgG binding domains, a six-histidine metal-binding domain, a cMyc epitope tag and a protease 3C cleavage site (Rubio *et al.*, 2005). Both groups have successfully purified protein complexes from plants using these expression vectors.

Plant destination vectors for modular assembly of transgenes

Recently, Invitrogen has expanded its repertoire of recombination sites in order to allow multiple gene elements to be recombined simultaneously into a destination vector. This modular approach allows one to choose among various promoters, reporter genes or epitope tags in entry vectors and then recombine these into a destination vector that will piece the elements together in the correct order. Karimi *et al.* have embraced this new technology to generate plant destination vectors bearing multi-site Gateway cassettes (Karimi *et al.*, 2005) (Figure 1h).

pEarleyGate vectors

We have designed a large set of Gateway-compatible plant destination vectors that are useful for epitope-tagging proteins of interest. As a prelude to designing Gateway-compatible epitope-tagging vectors, we conducted an evaluation of four epitope tag/antibody combinations in a variety of commonly studied plant species. We spiked total leaf protein extracts of tobacco (*Nicotiana tabacum*), *Arabidopsis thaliana*, maize (*Zea mays*), soybean (*Glycine max*), rice (*Oryza sativa*), tomato (*Lycopersicon esculentum*), and cotton (*Gossypium hirsutum*) (Figure 3a) with proteins displaying AcV5, HA, FLAG, and cMyc epitopes. Immunoblot detection of the tagged recombinant proteins was then conducted, as shown in Figure 3b–e. We found

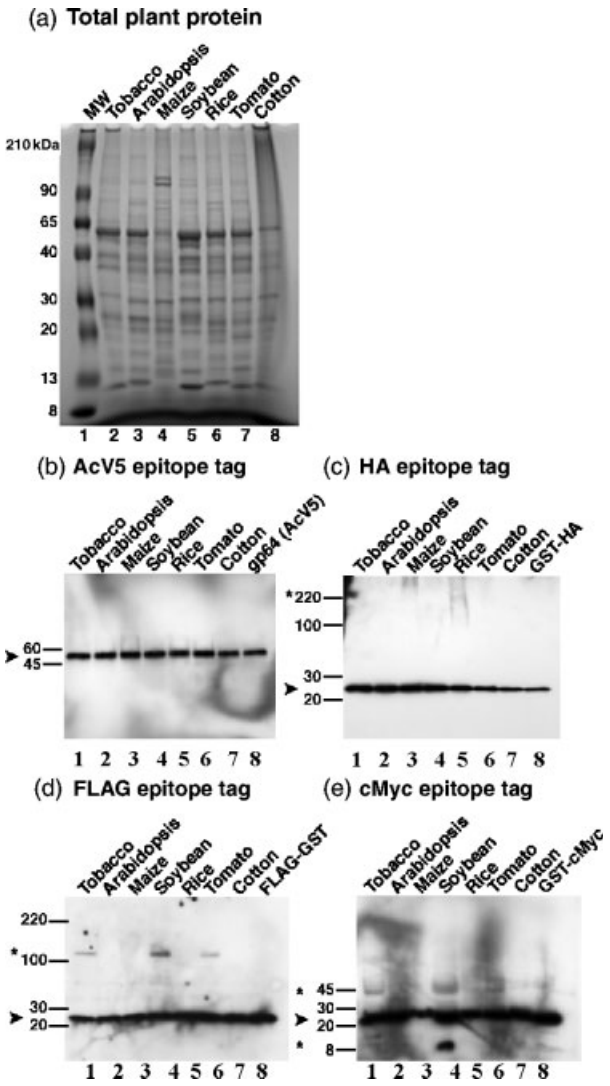


Figure 3. *In vitro* evaluation of AcV5, HA, FLAG and cMyc epitope detection in commonly studied plants.

(a) Total leaf protein (20 μg) extracted from tobacco (*Nicotiana tabacum*), *Arabidopsis thaliana*, maize (*Zea mays*), soybean (*Glycine max*), rice (*Oryza sativa*), tomato (*Lycopersicon esculentum*) or cotton (*Gossypium hirsutum*) was loaded in adjacent lanes of a 10–20% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel (Invitrogen). Following electrophoresis, the gel was stained using EZBlue Gel Staining Reagent (Sigma-Aldrich) to demonstrate that equivalent amounts of protein were loaded in each lane.

(b–e) Immunoblot detection of epitope-bearing proteins spiked into tobacco, *A. thaliana*, maize, soybean, rice, tomato or cotton protein samples. Total leaf protein (20 μg) was spiked with either (b) 225 ng of total viral protein from the baculovirus *Autographa californica*, which bears the AcV5 epitope on its gp64 coat protein, (c) 100 ng of glutathione S-transferase (GST) fused to an HA tag (GST–HA), (d) 100 ng of GST fused to a FLAG tag (FLAG–GST) or (e) 1 μg of GST fused to a cMyc tag (GST–cMyc). In lane 8 of each gel, the epitope-tagged recombinant protein alone was loaded as a control. Proteins were subjected to electrophoresis, immunoblotting using commercially available antibodies recognizing the four epitopes and chemilumiscent detection. Asterisks indicate cross-reacting proteins.

that all four epitope tags were readily detected in all species tested, although in some species there was cross-reaction between the antibodies and endogenous proteins. For instance, the HA antibody (Figure 3c) interacted with some high-molecular-weight proteins in maize and rice, the FLAG M2 antibody (Figure 3d) cross-reacted with an endogenous protein of approximately 125 kDa in tobacco, soybean, and tomato, and the cMyc (Clone 9E10) antibody (Figure 3e) cross-reacted with an endogenous protein of ~10 kDa in soybean and a protein of ~45 kDa in tobacco and soybean.

Based on the results of Figure 3, we designed Gateway-compatible vectors that would add AcV5, HA, FLAG, or cMyc epitope tags to either the N- or C-termini of target proteins (see Figure 4). We also engineered a vector containing a TAP tag consisting of a calmodulin-binding peptide separated from two copies of a Protein A peptide (which will bind to IgG resin) by a TEV protease cleavage site (Rigaut *et al.*, 1999). pEarleyGate vectors 201–205 allow the addition of HA, FLAG, cMyc, AcV5 or TAP epitope tags to target proteins encoded by cloned cDNA sequences. These vectors make use of the enhanced cauliflower mosaic virus 35S promoter for strong constitutive expression of tagged proteins. A second set of pEarleyGate vectors, 301–304, allows the addition of HA, FLAG, cMyc or AcV5 sequences to the C-terminus of recombinant transgenes. Because these vectors contain no promoter, they are useful for cloning genomic fragments that include promoter sequences, introns and exons, with the tag being added to the last exon in lieu of the natural stop codon. A third set of pEarleyGate vectors were engineered to add both a fluorescent protein and an epitope or His tag to a target protein: pEarleyGate 101 will add YFP with an HA tag, pEarleyGate 102 adds CFP with an HA tag, and pEarleyGate 103 will add GFP with a His tag. The pEarleyGate 101–103 vectors generate C-terminal fusions to the fluorescent protein/epitope tag. pEarleyGate 104 adds an N-terminal YFP to targeted proteins but contains no epitope tag sequence.

All 14 pEarleyGate vectors are derived from pFGC5941 (http://www.chromDB.org), which was built using a pCAMBIA (http://www.cambia.org) binary vector backbone. pEarleyGate vectors support *Arabidopsis tumefaciens*-mediated stable transformation, and can be obtained from the Arabidopsis Biological Resource Center (http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm). Detailed information for pEarleyGate vectors, including maps and sequence information, is available at the Pikaard laboratory website (http://biology4.wustl.edu/pikaard/).

In vivo evaluation of pEarleyGate vectors

Detection of different epitope-tagged versions of the same target protein, expressed from pEarleyGate derived T-DNAs in transgenic *A. thaliana*, is shown in Figure 5. For this comparison, the open reading frame for HDA6, an *A. thaliana* histone deacetylase, was recombined into pEarleyGate 200-series vectors. Resulting N-terminal HA, FLAG, cMyc, or AcV5-tagged recombinant proteins or C-terminal TAP-tagged proteins were expressed from mRNAs driven by the cauliflower mosaic virus 35S promoter. Multiple transgenic *A. thaliana* lines were generated for each pEarleyGate construct. Leaf tissue from individual primary transformants was then homogenized in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer and boiled, and an aliquot of the resulting lysate was loaded in a single lane of an SDS–PAGE gel. Following electophoresis and immunoblotting, the recombinant proteins were detected using commercially available antibodies recognizing the different epitope tags. As shown in Figure 5, HA, FLAG, cMyc, AcV5 and TAP tagged HDA6 proteins were detected in multiple independent lines, with expression levels varying from line to line. Relatively low background cross-reaction with endogenous proteins was observed for all antibodies tested, consistent with the prior spiking experiments. Smaller products detected in protein extracts of plants expressing full-length tagged proteins but not detected in non-transgenic controls are presumably cleavage products or incomplete translation products derived from the transgenes.

Use of epitope tags for affinity purification

To evaluate the usefulness of pEarleyGate vectors for production of recombinant proteins that can be affinity-purified by virtue of their epitope tags, we extracted total soluble protein from *A. thaliana* lines overexpressing HDA6 tagged with FLAG, HA, or cMyc epitopes. Anti-HA, FLAG, or cMyc antibodies conjugated to agarose beads were then used to capture the tagged proteins. For each epitope tag tested, HDA6 protein was effectively affinity-captured and greatly enriched in bead-associated fractions as compared with input extracts (Figure 6a).

Interestingly, elution of the protein from the matrix using excess epitope peptides appears to be more difficult for some antibody–epitope combinations than for others. For instance, FLAG-tagged HDA6 could be eluted using a high concentration of competing peptide, but cMyc and HA (data not shown) tagged proteins were not eluted using similar conditions. The latter tagged proteins were only eluted under denaturing conditions in SDS–PAGE sample buffer (Figure 6b).

We were also interested in determining if pEarleyGate epitope-tagging vectors are useful for immunolocalization experiments. For this set of experiments we recombined the cDNA sequence for *HDT1*, a histone deacetylase known to localize to the nucleolus when fused to GFP or YFP (Lawrence *et al.*, 2004; Zhou *et al.*, 2004), into pEarleyGate 200-series vectors. As shown in Figure 7, immunolocalization of the cMyc epitope reveals that the

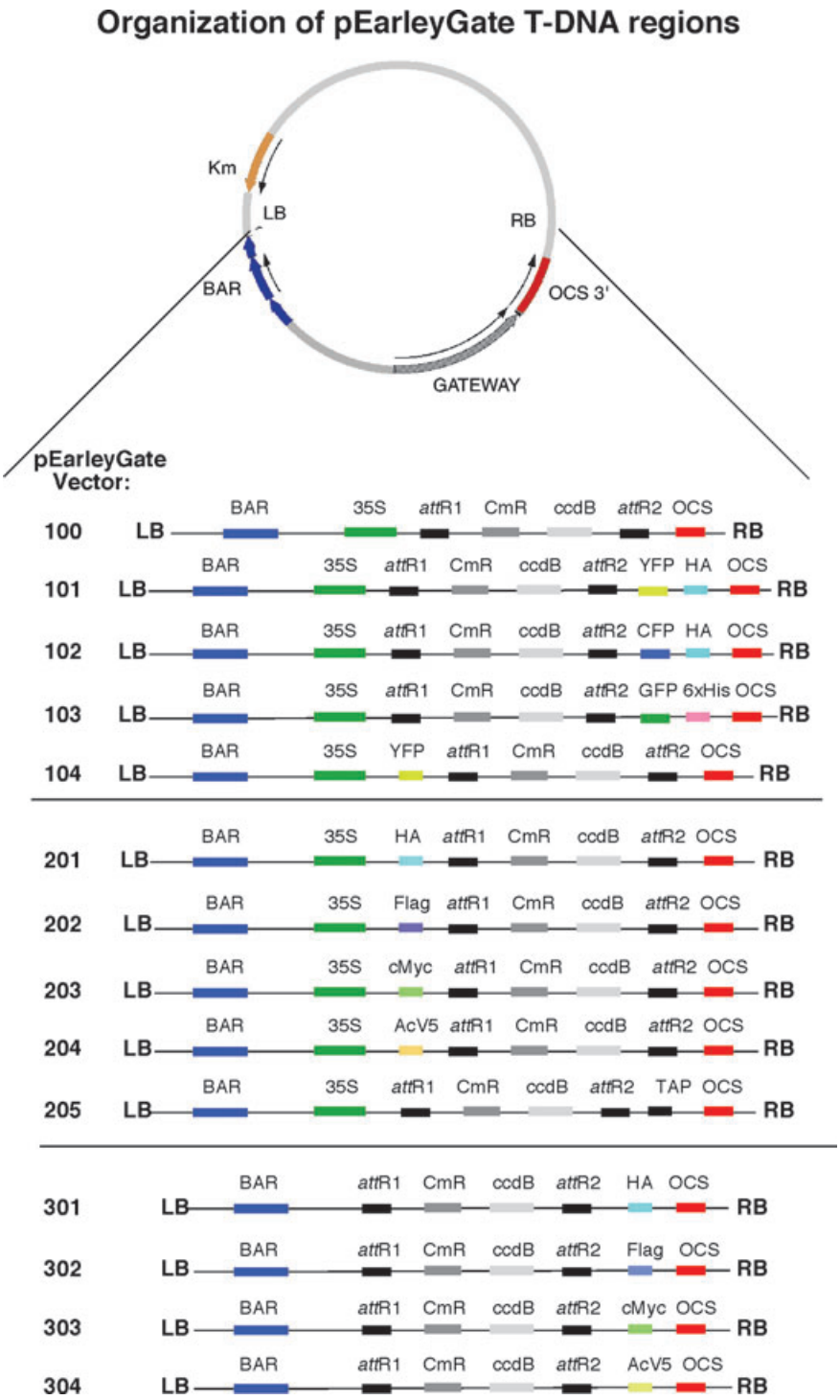


Figure 4. pEarleyGate plant transformation vectors. The pEarleyGate vectors are derived from pFGC5941 (http://www.chromDB.org), which was built using a pCAMBIA (http://www.cambia.org/) plasmid backbone. As a result, all of the pEarleyGate plasmids are binary vectors that will replicate in both *Escherichia coli* and *Agrobacterium tumefaciens* and have left border (LB) and right border (RB) sequences for *Agrobacterium*-mediated T-DNA transfer.

The organization of the T-DNAs for each of the various pEarleyGate vectors is shown. The Gateway cassettes in each vector include *att*R1, a chloramphenicol resistance gene (*CmR*), the *ccdB* killer gene and *att*R2. 35S, the cauliflower mosaic virus 35S promoter and its upstream enhancer. OCS, the 3′ sequences of the octopine synthase gene, including polyadenylation and presumptive transcription termination sequences. BAR, the Basta herbicide resistance gene for selection of transgenic plants. Km, the bacterial kanamycin resistance gene within the plasmid backbone. Different pEarleyGate vectors allow engineering and expression of proteins fused in frame with HA, FLAG, cMyc, AcV5 or tandem affinity purification (TAP) tags and/or yellow, green or cyan fluorescent proteins (YFP, GFP or CFP, respectively) at either the amino-terminal or carboxy-terminal end of the target proteins.

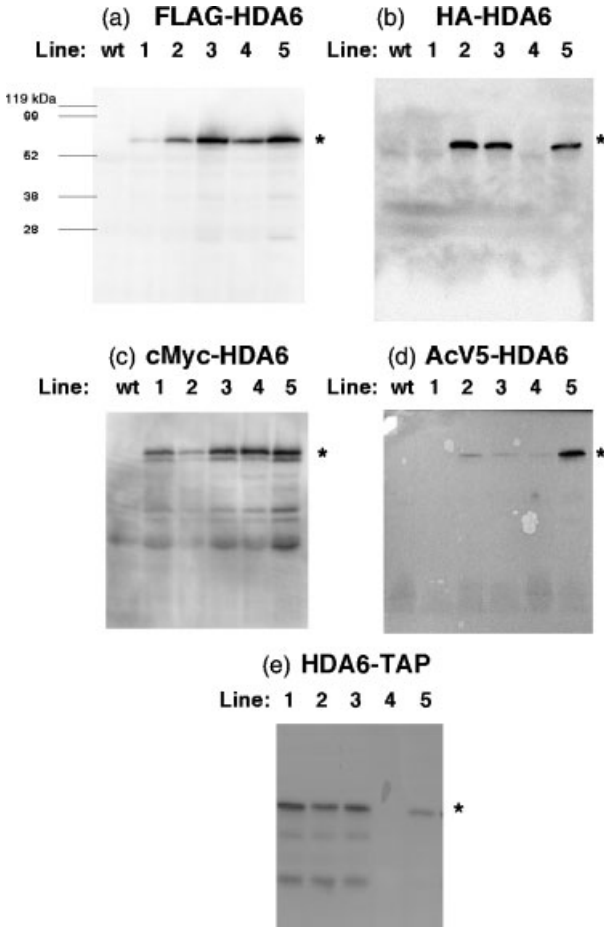


Figure 5. Immunoblot detection of epitope-tagged recombinant proteins expressed from pEarleyGate-derived T-DNAs in *Arabidopsis thaliana*. The open reading frame of *HDA6* was recombined into pEarlyGate 202, 201, 203, 204 or 205 to generate FLAG, HA, cMyc, AcV5, or tandem affinity purification (TAP)-tagged HDA6 fusion proteins, respectively. For each construct, leaf tissue from five independent Basta-resistant T1 plants (lanes 1–5) or a non-transformed control (wt) plant was homogenized in sodium dodecyl sulfate (SDS) sample buffer and equal aliquots were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 12.5% Tris-glycine gel. Proteins were transferred to nitrocellulose or PVDF membrane and epitope-tagged proteins were detected using: (a) anti-AcV5 monoclonal antibody (diluted 1:2000) followed by anti-mouse immunoglobulin G (IgG)–horseradish peroxidase (HRP) secondary antibody (diluted 1:2000), or (b) anti-HA–HRP monoclonal antibody (diluted 1:3000), or (c) anti-FLAG-AP M2 monoclonal antibody (diluted 1:1000), or (d) anti-cMyc–alkaline phosphatase (AP) monoclonal antibody (diluted 1:1000), or (e) peroxidase-conjugated anti-IgG (diluted 1:2000). Protein–antibody complexes were visualized by chemi-luminescent detection of AP or HRP activity. Asterisks indicate full-length epitope-tagged HDA6.

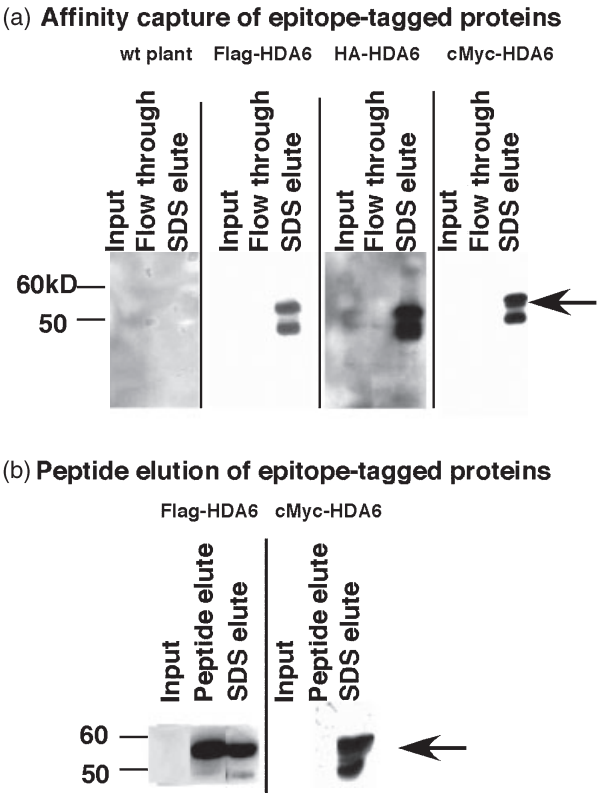


Figure 6. Affinity purification of FLAG, HA, or cMyc-tagged HDA6 expressed in *Arabidopsis thaliana* transgenic plants.
(a) *A. thaliana* plants expressing FLAG, HA, or cMyc-tagged HDA6 were homogenized in extraction buffer and incubated with anti-FLAG, anti-HA or anti-cMyc antibodies conjugated to agarose beads. Beads and bound proteins were then washed extensively with extraction buffer and bound proteins were eluted by boiling in sodium dodecyl sulfate (SDS) sample buffer. Equal aliquots of the input homogenate, wash (flow-through) and eluted proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and recombinant proteins were detected by immunoblotting using anti-FLAG, anti-HA or anti-cMyc antibodies. Arrows indicate full-length epitope-tagged HDA6.
(b) Peptide elution of affinity-captured proteins works better for some epitope tags than for others. FLAG- or cMyc-tagged HDA6 affinity captured on agarose beads was first incubated with FLAG or cMyc peptide under non-denaturing conditions and beads were subsequently boiled in SDS sample buffer. Aliquots of the input, peptide-eluted or SDS-eluted fractions were subjected to SDS–PAGE and recombinant proteins were detected by immunoblotting using anti-FLAG or anti-cMyc antibodies. Arrows indicate full-length epitope-tagged HDA6. Note that FLAG-tagged HDA6 could be peptide-eluted but cMyc-tagged protein was not eluted from beads using cMyc peptide.

tagged HDT1 protein is detected in the nucleolus of transgenic plants, as expected.

In vivo evaluation of pEarleyGate fluorescent protein fusion vectors

pEarleyGate vectors designed for fusing target proteins to GFP, YFP or CFP include an epitope tag fused in frame with the fluorescent protein. Their design allows the vectors to be used for *in vivo* localization of resulting fluorescent fusion proteins, for immunolocalization of the protein in fixed cells by virtue of the epitope tag or for affinity purification or detection of the protein on immunoblots. As a test of the pEarleyGate fluorescent protein fusion vectors, we recombined the *HDT1* cDNA into pEarleyGate 101. As expected, the HDT1-YFP-HA fusion protein localizes to the nucleolus, as can be deduced by comparing the fluorescence signal with the differential interference contrast (DIC)

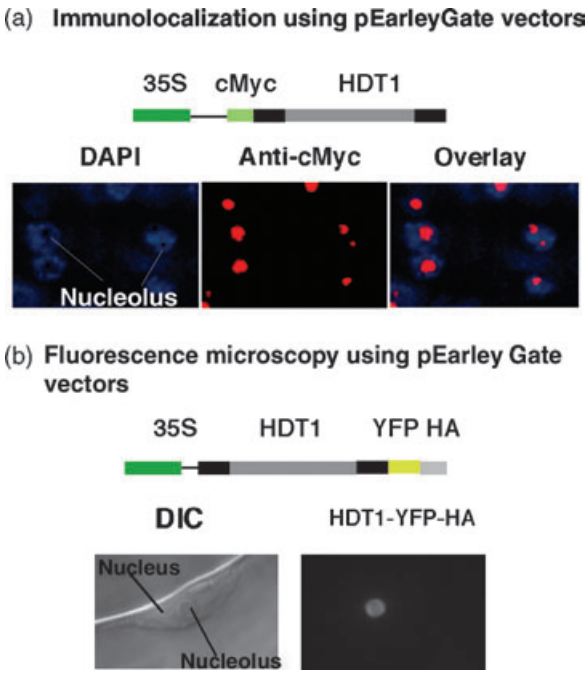


Figure 7. Use of pEarleyGate vectors for protein localization experiments.
(a) Immunolocalization of cMyc-tagged HDT1 expressed using pEarleyGate 203. HDT1 localizes to the nucleolus (n), which corresponds to the 4′,6-diamidino-2-phenylindole-negative region(s) of the nuclei.
(b) Localization of HDT1-YFP-HA fusion protein expressed using pEarleyGate 101. The protein was localized by virtue of yellow fluorescent protein (YFP) fluorescence. The nucleus and nucleolus are clearly visible in the image obtained by differential interference contrast (DIC) microscopy.

image (Figure 7b). Upon boiling leaf tissue in SDS–PAGE sample buffer, and subjecting extracted proteins to SDS–PAGE and immunoblotting using anti-HA antibody, the HDT1-YFP-HA fusion protein is also readily detected by virtue of its epitope tag (data not shown). Collectively, these data demonstrate that pEarleyGate 101–103 can be useful for detecting proteins both *in situ* and following fractionation and immunoblotting.

Concluding remarks

Gateway technology is increasingly used to facilitate proteomic analyses (Gong *et al.*, 2004; Koroleva *et al.*, 2005; Pendle *et al.*, 2005; Reboul *et al.*, 2003; Tian *et al.*, 2004) and efforts are ongoing to clone the *A. thaliana* ORFeome (the comprehensive collection of full-length cDNAs) into Gateway pENTR vectors (Gong *et al.*, 2004; http://www.evry.inra.fr/public/projects/orfeome/orfeome.html). One can shuttle these ORFs into the various destination vectors now available. We anticipate that the pEarleyGate vectors will be a useful addition to the sets of Gateway-compatible vectors already available to the plant community for protein overexpression, gene silencing, protein localization and promoter analysis.

Experimental procedures

Notes on the use of pEarleyGate destination vectors

(i) The pENTR/D-TOPO vector that we use in most of our recombination reactions contains the same bacterial selection marker as the pEarleyGate vectors (kanamycin resistance). To prevent transformation of bacteria with the pENTR plasmid following the recombination reaction, we cut the pENTR vector bearing the target sequence of interest with a restriction endonuclease that cleaves within the pENTR backbone but does not cut within the target sequence. We often use *Mlu*I, which cuts twice within the pENTR backbone. Most other Gateway-compatible destination vectors have different selectable markers, in which case the pENTR plasmid does not need to be cut before the recombination reaction. Alternatively, one could make use of a pDONR vector that has an antibiotic resistance marker other than kanamycin.

(ii) Before recombining the sequence of interest into the pEarleyGate vectors, we typically gel-purify the digested fragment that contains the sequence of interest flanked by the *att*L sites. However, the recombination reaction also works with cleaved DNA that is purified using a commercial DNA clean-up kit.

(iii) We recombine ~100 ng of pEarleyGate plasmid DNA with ~100 ng of pENTR fragment using the LR clonase reaction mix (Invitrogen). We find that the concentration of the two fragments can vary without disrupting the success rate of the recombination. We have also found that clonase reactions can be scaled down to half-reactions without jeopardizing successful recombination events, which reduces the cost per reaction.

(iv) After the recombination reaction, we treat the reaction with proteinase K to digest the clonase enzymes, and transform the resulting reaction into a *ccdB*-sensitive strain of *E. coli* (we typically use DH5-alpha). We select for positive clones by plating transformation reactions on LB medium that contains 50 μg ml^{-1} kanamycin.

Detailed protocols for capturing target sequences in entry vectors and transferring them to destination vectors are available at Invitrogen's website (http://www.invitrogen.com).

Plant Material

Arabidopsis thaliana ecotype Columbia, *Z. mays*, *O. sativa*, *G. max* and *L. esculentum* were grown for 4 to 6 weeks under long-day conditions (16 h light/8 h dark) at room temperature using fluorescent light illumination. *N. tabacum* and *G. hirsutum* were grown for 4 weeks at ~25°C on a 14 h light/10 h dark cycle. For immunoblot analysis of epitope-tagged constructs and immunoprecipitation experiments, *A. thaliana* plants were grown for 2 to 3 weeks under long-day conditions. For fluorescent protein analyses, transgenic *A. thaliana* seeds were germinated on sterile semi-solid Murashige–Skoog medium (Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 1% sucrose (pH 5.8), and plants were examined after 2 weeks of growth.

Epitope tag sequences

The FLAG epitope sequence used in this study is DYKDDDDK; the HA epitope is YPYDVPDYA; the cMyc epitope is EQKLISEEDL; the AcV5 epitope is SWKDASGWS, and the TAP tag sequence is

EKRRWKKNFIAVSAANRFKKISSSGALDYDIPTTASENLYFQGELKTA-ALAQHDEAVDNKFNKEQQNAFYEILHLPNLNEEQRNAFIQSLKDDPS-QSANLLAEAKKLNDAQAPKVDNKFNKEQQNAFYEILHLPNLNEEQR-NAFIQSLKDDPSQSANLLAEAKKLNGAQAPKVDANSAGKST (Rigaut *et al.*, 1999).

Epitope-tagged protein spiking experiments

Recombinant proteins used in the protein spiking study were cloned and expressed in bacterial expression vectors based on the MAC vector backbone (Sigma-Aldrich). Inserts were generated by PCR and directionally cloned using the Director Universal PCR kit (Sigma-Aldrich). Recombinant epitope-tagged proteins FLAG–GST, GST–cMyc, and GST–HA were expressed in *E. coli* strain BL21-DE3 and affinity-purified using glutathione affinity resin (Sigma-Aldrich). Proteins were quantified by the method of Bradford (Bradford, 1976) using commercially available Bradford Reagent (Sigma-Aldrich).

Total leaf protein was extracted from 100 mg of fresh leaf tissue using the Plant Total Protein Extraction Kit (Sigma-Aldrich) supplemented with 1:100 [volume/volume (v/v)] diluted plant protease inhibitor cocktail (Sigma-Aldrich). The protein concentration was determined by the method of Bradford (Bradford, 1976). Total protein (20 μg) was then spiked with 100 ng of FLAG–GST, 100 ng of GST–HA, 1 μg of GST–cMyc, or 225 ng of *Autographa californica* total protein and subjected to SDS–PAGE, electroblotting to Hybond-ECL nitrocellulose (Amersham Biosciences, Piscataway, NJ, USA) or PVDF (Millipore, Billerica, MA, USA) membrane, and probing with appropriate antibodies using standard methods (Fritze and Anderson, 2000). Anti-FLAG M2® monoclonal antibody–alkaline phosphatase conjugate, anti-HA monoclonal antibody–peroxidase conjugate (Clone HA-7), anti-cMyc monoclonal antibody–alkaline phosphatase conjugate (clone 9E10), anti-mouse IgG (whole molecule)–alkaline phosphatase conjugate, and peroxidase-conjugated anti-peroxidase were all from Sigma-Aldrich; anti-*Autographa californica* gp64 protein monoclonal antibody (clone AcV5) was from eBioscience (San Diego, CA, USA).

For Western blot analysis of protein spiking experiments, the following dilutions of antibodies were used. Anti-AcV5 monoclonal antibody was diluted 1:2000 prior to incubation with the blot and was detected, after washing, using 1:30 000-diluted anti-mouse IgG (whole molecule)–alkaline phosphatase (AP) conjugate as the secondary antibody. Other epitopes were detected following a single incubation with AP- or horseradish peroxidase (HRP)-conjugated primary antibodies. Final dilutions for the antibodies were: anti-HA–HRP, 1:10 000; anti-FLAG M2–AP, 1:10 000; and anti-cMyc–AP, 1:50 000. Chemiluminescent detection of alkaline phosphatase (AP) or peroxidase (HRP) activity was performed using CDP-Star Chemiluminescent substrate and Chemiluminescent Peroxidase substrate, respectively (Sigma-Aldrich).

Construction of pEarleyGate plasmid vectors

pEarleyGate 100–105. To create pEarleyGate 100, the Gateway cassette was amplified by PCR from the Reading Frame B DNA fragment (purchased from Invitrogen) using the following primers: forward 5′-cgcgctcgagatcacaagtttgtacaaaaaagc-3′ and reverse 5′-gccctaggcaccactttgtacaagaaagc-3′. The resulting PCR product was digested with *Xho*I and *Avr*II and ligated (Rapid DNA Ligation Kit; Roche, Mannheim, Germany) into pFGC5941 (http://www.ChromDB.org), replacing its *Xho*I to *Avr*II fragment. To create pEarleyGate101 and 102, YFP and CFP were amplified by PCR using primers forward 5′-tgcctagggtgagcaagggcgaggagc-3′ and reverse 5′-tcttaattaagcgtaatctggaacatcgtatgggtatctagatccggtggatcc-3′. Resulting PCR products were digested with *Avr*II and *Pac*II and inserted into the adjacent *Avr*II and *Pac*II sites of pEarleyGate 100. To create pEarleyGate 104, YFP was excised from pCAM-35S-EYFP-C1 (Fritze and Anderson, 2000) using *Bam*HI and *Nco*I and ligated into the *Bam*HI and *Nco*I sites of pFGC5941, replacing its *Bam*HI–*Nco*I fragment. The Gateway cassette was then added by PCR amplifying the Reading Frame B cassette using primers forward 5′-cgagatctatcacaagtttgtacaaaaaagc-3′ and reverse 5′-cgcagatctcaccactttgtacaagaaagc-3′ and ligating the resulting PCR product into the *Nco*I and *Avr*II sites of the plasmid that had been converted to blunt ends by treatment with T4 DNA polymerase (NEB) and 10 mM dNTPs. To create pEarleyGate 103, the GFP–6 × His fragment of pCAMBIA 1302 was amplified by PCR, cut with *Xho*I and *Avr*II, and ligated into pFGC5941, replacing its *Xho*I to *Avr*II fragment. The Gateway cassette was then added by amplifying the Reading Frame B DNA fragment by PCR using the primers forward 5′-cgcgctcgagatcacaagtttgtacaaaaaagc-3′ and reverse 5′-cgcgctcgagcaccactttgtacaagaaag-3′, cutting with *Xho*I and ligating the resulting PCR fragment into the *Xho*I site of the plasmid.

pEarleyGate 201–205. Gateway cassettes with adjacent epitope tag sequences were amplified by PCR using the Invitrogen Reading Frame B sequence. Forward primers adding HA, FLAG, cMyc, or AcV5 epitope tags to Gateway cassette sequences were: HA, 5′-acccatacgatgttccagattacgctatcacaagtttgtacaaaaaagc-3′; FLAG, 5′-gactacaaagacgatgacgacaaaatcacaagtttgtacaaaaaagc-3′; cMyc, 5′-gaacagaaagtgatctctgaagaagatctgatcacaagtttgtacaaaaaagc-3′; AcV5, 5′-tcttggaaagatgcgagcggctggtctatcacaagtttgtacaaaaaagc-3′. An identical reverse primer, 5′-aattaactctctagactcacctaggc-3′, was used for all PCR reactions. Resulting PCR products were cloned into pFGC5941 that had been digested with *Nco*I and *Avr*II and treated with T4 DNA polymerase and 10 mM dNTPs to generate blunt ends. To create pEarleyGate 205, the TAP fragment of pBM3947 was amplified by PCR using primers forward 5′-cctagggagatggaaaagagaagatg-3′ and reverse 5′-gccttaattaatcaggttgacttcccc-3′, cut with *Avr*II and *Pac*I and ligated into pEarleyGate100.

pEarleyGate 301–304. Gateway cassettes with adjacent epitope tag sequences were amplified by PCR using the Invitrogen Reading Frame B sequence. Reverse primers adding HA, FLAG, cMyc, or AcV5 epitope tags to Gateway cassette sequences were: HA, 5′-tcaagcgtaatctggaacatcgtatgggtacaccactttgtacaagaaagc-3′; FLAG, 5′-tcatttgtcgtcatcgtctttgtagtccaccactttgtacaagaaagc-3′; cMyc, 5′-tcacagatcttcttcagagatcagtttctgttccaccactttgtacaagaaagc-3′; AcV5, 5′-tcaagaccagccgctcgcatctttccaagacaccactttgtacaagaaagc-3′. An identical forward primer, 5′-gaattctgcagtcgacgg-3′, was used for all PCR reactions. Resulting PCR products were ligated into pFGC5941 which had been digested with *Eco*RI and *Avr*II and treated with T4 DNA polymerase and 10 mM dNTPs to generate blunt ends.

All ligation reactions including the Gateway cassette were transformed into *E. coli* DB3.1 cells (Invitrogen), which are resistant to the *ccdB* gene. Positive clones were selected on LB plates containing 34 μg ml^{-1} chloramphenicol.

Recombination of target sequences into pEarleyGate plant expression vectors

HDA6 and *HDT1* coding sequences, either with or without their natural stop codon, were amplified from cloned cDNAs by PCR

using Platinum Pfx polymerase (Invitrogen) and the following primers: *HDA6* forward 5′-caccatggaggcagacgaaagc-3′ and reverse 5′-ctagagagctgggacactgagc-3′; *HDT1* (no stop) forward 5′-caccatggagttctggggaattg-3′ and reverse 5′-cttggcagcagcgtgcttgg-3′; *HDT1* (stop) forward 5′-caccatggagttctggggaattg-3′ and reverse 5′-tcacttggcagcagcgtgc-3′. The resulting PCR products were captured by topoisomerase-mediated cloning into the paENTR/D-TOPO vector (Invitrogen). Entry clones containing *HDT1* and *HDA6* sequences, pENTR-*HDA6* and pENTR-*HDT1*, were cut with *Mlu*I to linearize the pENTR plasmid in order to prevent subsequent transformation of *E. coli* by the entry vector rather than (or in addition to) the pEarleyGate destination vector (see notes on the use of pEarleyGate vectors, above). The DNA fragment containing the *HDA6* sequence flanked by *att*L recombination sites was recombined into the pEarleyGate 201, 202, 203, 204, and 205 plasmids using LR clonase (Invitrogen). The DNA fragment containing *HDT1* without a stop codon was recombined into pEarleyGate 101 to form a C-terminal YFP–HA fusion and the DNA fragment containing pENTR-*HDT1* with a stop codon was recombined into pEarleyGate 203 to form a N-terminal cMyc fusion. Recombined plasmids were transformed into *E. coli* DH5-alpha cells. Positive clones were selected on kanamycin LB plates. Recombinant plasmids were then transformed into *A. tumefaciens* strain LBA 4404 for subsequent plant transformation.

Plant transformation and detection of epitope-tagged recombinant proteins

A. tumefaciens-mediated transformation of *A. thaliana* ecotype Columbia was accomplished by using the floral dip technique (Bechtold and Pelletier, 1998) as modified by Clough and Bent (Clough and Bent, 1998).

A single leaf from plants transformed with pEarleyGate vectors was homogenized in 400 μl of SDS–PAGE sample buffer [50 mM Tris (pH 6.8), 6% glycerol, 2% SDS, 100 mM dithiothreitol (DTT), and 0.01% bromophenol blue] and boiled for 5 min. Samples were centrifuged at 16 000 ***g*** for 10 min. A volume of 20 μl of supernatant was loaded onto SDS–PAGE gel and epitope-tagged proteins were detected by immunoblotting. Antibody dilutions used for detection of *in planta* expressed epitope-tagged proteins by Western blot analysis are included in the legend of Figure 5.

Affinity purification experiments

Above-ground tissues of 3-week-old *A. thaliana* plants expressing HA, FLAG, cMyc, or AcV5 tagged HDA6 transgenes were harvested and ground to a fine powder in liquid nitrogen. Two volumes [weight/volume (w/v/)] of Cell Lytic P (Sigma) solution, amended to include 1:100 (v/v) diluted plant-specific protease inhibitor cocktail (Sigma-Aldrich) and 1 mM phenylmethylsulfonyl fluoride (PMSF), was then mixed with the powder. Homogenates were filtered through four layers of miracloth (Calbiochem, San Diego, CA, USA) and subjected to centrifugation at 6000 ***g*** for 15 min. The supernatant containing epitope-tagged HDA6 was incubated with anti-HA, anti-cMyc or anti-FLAG-conjugated agarose (all from Sigma-Aldrich) for 1 h at 4°C. The conjugated agarose resins were washed twice with Cell Lytic P extraction buffer and proteins were eluted with SDS–PAGE sample buffer (50 mM Tris–HCl, pH 6.8, 6% glycerol, 2% SDS, 100 mM DTT and 0.01% bromophenol blue) or Cell Lytic P buffer containing 3× FLAG peptide (200 μg ml^{-1}). Samples were subjected to electrophoresis on an SDS–PAGE gel, transferred to PVDF membrane and analyzed by immunoblotting with the appropriate antibody.

Analysis of fluorescent tags and immunolocalization experiments

Root tissue expressing HDT1-YFP-HA was imaged using a Zeiss M2Bio microscope equipped with a Zeiss Axiocam digital camera and a Nikon Eclipse E600 fluorescence microscope with a Q Imaging Retiga EX digital camera. Fluorescence microscopy and immunolocalization experiments were performed as previously described (Lawrence *et al.*, 2004; Onodera *et al.*, 2005).

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