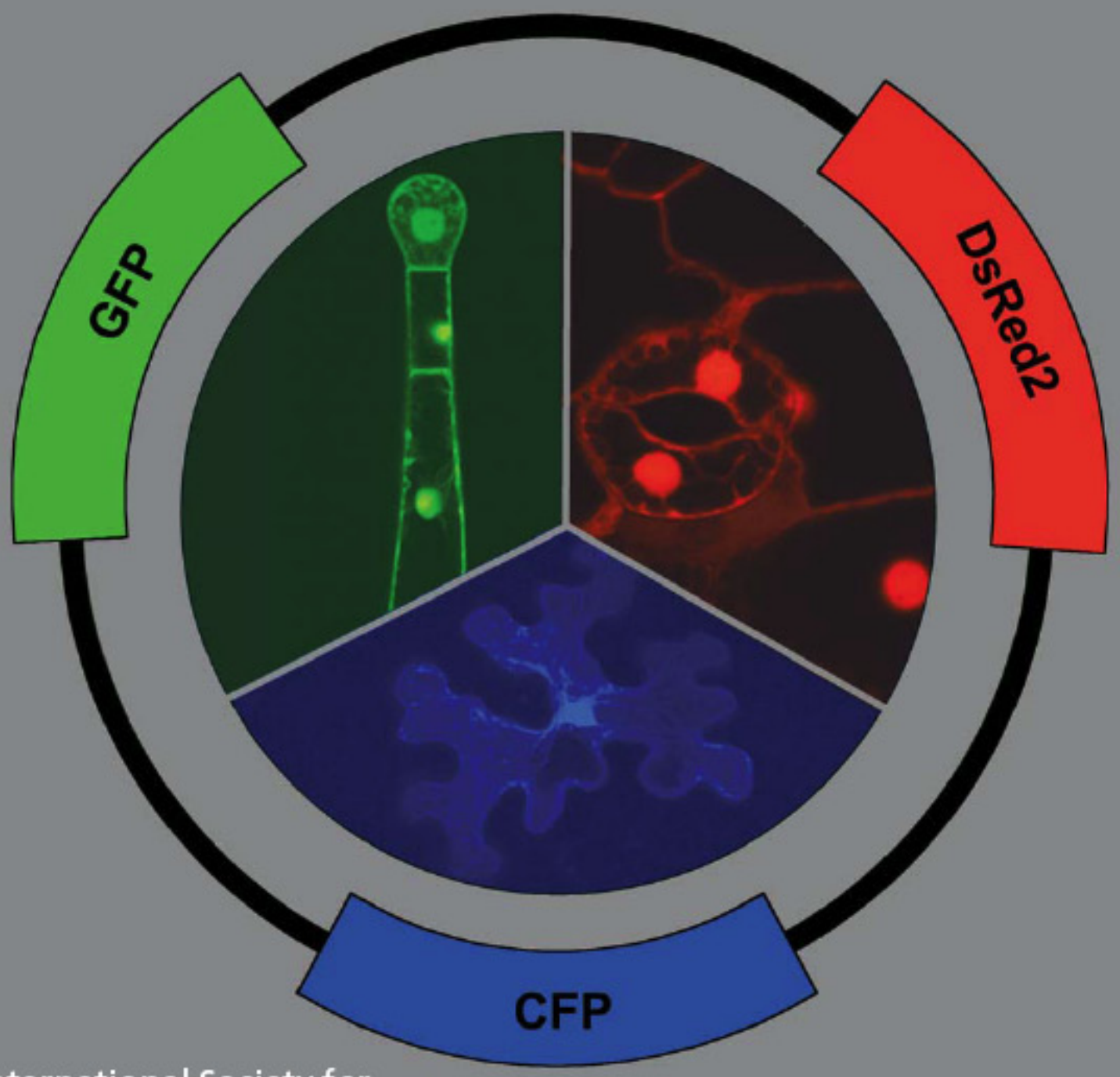
Volume 57, No. 4, March (I) 2005

ISSN 0167-4412
CODEN PMBIDB

PLANT MOLECULAR BIOLOGY



International Society for Plant Molecular Biology

Springer

Coexpression of multiple autofluorescently-tagged proteins

Plant Molecular Biology (2005) 57:503–516
DOI 10.1007/s11103-005-0340-5

pSAT vectors: a modular series of plasmids for autofluorescent protein tagging and expression of multiple genes in plants

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Received 7 December 2004; accepted in revised form 7 January 2005

Key words: autofluorescent proteins, multiple gene expression, plasmids

Abstract

Autofluorescent protein tags represent one of the major and, perhaps, most powerful tools in modern cell biology for visualization of various cellular processes *in vivo*. In addition, advances in confocal microscopy and the development of autofluorescent proteins with different excitation and emission spectra allowed their simultaneous use for detection of multiple events in the same cell. Nevertheless, while autofluorescent tags are widely used in plant research, the need for a versatile and comprehensive set of vectors specifically designed for fluorescent tagging and transient and stable expression of multiple proteins in plant cells from a single plasmid has not been met by either the industrial or the academic communities. Here, we describe a new modular satellite (SAT) vector system that supports N- and C-terminal fusions to five different autofluorescent tags, EGFP, EYFP, Citrine-YFP, ECFP, and DsRed2. These vectors carry an expanded multiple cloning site that allows easy exchange of the target genes between different autofluorescence tags, and expression of the tagged proteins is controlled by constitutive promoters, which can be easily replaced with virtually any other promoter of interest. In addition, a series of SAT vectors has been adapted for high throughput Gateway recombination cloning. Furthermore, individual expression cassettes can be assembled into *Agrobacterium* binary plasmids, allowing efficient transient and stable expression of multiple autofluorescently tagged proteins from a single vector following its biolistic delivery or *Agrobacterium*-mediated genetic transformation.

Introduction

Since the discovery of the jellyfish green autofluorescent protein (GFP) (Chalfie *et al.*, 1994), autofluorescent protein tags have emerged as one of the major and, perhaps, most powerful tools for visualization of cellular structures, intra- and intercellular localization of proteins, monitoring gene expression, studies of *in vivo* protein–protein interactions and cellular dynamics, and other important biological experimentation (reviewed in refs. Day *et al.*, 2001; Hanson and Kohler, 2001; Gerlich *et al.*, 2003; March *et al.*, 2003; Miyawaki, 2003). In addition, technical advances in confocal microscopy (Harms *et al.*, 2001; Michalet *et al.*, 2003; Zemanova *et al.*, 2003; Zimmermann *et al.*, 2003) and its falling costs have made this approach a common and, in fact, often the only possible methodology for studies of many cellular processes. The versatility and utility

of autofluorescent protein (AFP) tags was advanced by development of multicolor spectral variants of GFP, such as yellow (YFP), cyan (CFP) and blue (BFP), as well as isolation of a red autofluorescent protein DsRed from coral (Matz *et al*., 1999). These already improved tags were further enhanced, for example, by accelerating their maturation rate (Venus YFP, Nagai *et al*., 2002), reducing aggregation (DsRed2, developed by Clontech, Palo Alto, CA, USA), reducing sensitivity to chloride ions and pH (Citrine-YFP and Venus-YFP, Griesbeck *et al*., 2001; Nagai *et al*., 2002), providing UV excitability (Sapphire-GFP, Tsien, 1998), and eliminating the need for multimerization (monomeric variant of DsRed, mRFP1, Campbell *et al*., 2004).

Development of AFP tags with different excitation and emission spectra allowed their simultaneous use for detection of two or more events in the same cell, i.e., fluorescent resonance energy transfer (FRET) between DsRed and GFP or CFP (Erickson *et al*., 2003) or YFP and CFP (Tsien and Miyawaki, 1998; Pollok andHeim, 1999) during protein–protein interaction, or simply for labeling different proteins in the same cell. For example, dual labeling with GFP/DsRed (e.g., Yang *et al*., 2003), BFP/GFP (e.g., Philipps *et al*., 2003), GFP/YFP (e.g., Rehm *et al*., 2003), CFP/YFP (e.g., Galperin and Sorkin, 2003), and even triple labeling with GFP/BFP/DsRed (e.g., Finley *et al*., 2001) have been extensively used in mammalian systems following introduction of several plasmids into the same cell. As exemplified by the Living Color™ family of plasmids (Clontech), this demand for multicolor AFP tags has been met by commercialization of numerous and versatile gene fusion vectors designed for expression in mammalian, bacterial, and yeast cells.

The use of AFP tags is also rapidly expanding to many areas of plant research (Hanson and Kohler, 2001; Stewart, 2001; reviewed in Berg, 2004), from protein localization (e.g., Goodin *et al*., 2002) to protein–protein interactions (e.g., Hink *et al*., 2002; Bracha-Drori *et al*., 2004; Tzfira *et al*., 2004; Walter *et al*., 2004) to global tagging of full-length gene products *in planta* (Tian *et al*., 2004). Recently, construction of several plasmids has been reported for protein AFP tagging and expression as well as for coexpression of two autofluorescently tagged proteins in plant cells. One such system, for example, describes a set of Gateway cloning vectors useful for N- or C-terminal fusions to GFP (Curtis and Grossniklaus, 2003) while another similar system of *Agrobacterium* Gateway binary plasmids (Karimi *et al*., 2002) was later modified to allow protein fusions to the red fluorescent protein RFP (Campbell *et al*., 2004) and coexpression of GFP- and RFP-tagged proteins in plant cells (Van Damme *et al*., 2004). Also, a set of additional variants of the Gateway cloning binary vectors (Karimi *et al*., 2002) is described on the Internet (www.psb.ugent.be/gateway). Although these systems allow Gateway-based AFP tagging, each of their plasmids is specifically designed for a unique and limited task (e.g., promoter analysis or constitutive expression of N- or C-terminal AFP fusions), and the tagged protein expression cassettes can not be easily mobilized between plasmids while the plasmids themselves are nearly impossible to manipulate for adjustment for specific research requirements. Furthermore, a recently reported system, that allows insertion of up to six expression cassettes into a single plasmid (Goderis *et al*., 2002), lacks regulatory elements necessary for gene expression (i.e., promoters and terminators), and contains only a very limited multiple cloning site (MCS).Thus, there is a clear need for a new versatile AFP tagging and multiple gene expression system that avoids these flaws of the existing technologies.

Here, we describe a new modular system of vectors that support N- or C-terminal fusions to five different autofluorescent tags (EGFP, EYFP, Citrine-YFP, ECFP, and DsRed2) expressed from constitutive promoters. These vectors contain an expanded MCS, and allow easy exchange of the target genes between autofluorescence tag cassettes as well as replacement of promoter and terminator sequences. Importantly, individual protein expression cassettes can be assembled into a single vector, allowing efficient transient or stable expression, potentially from different regulatory elements, of multiple autofluorescently-tagged proteins following biolistic delivery or *Agrobacterium*-mediated genetic transformation. We believe that our vector system, useful to most members of the research community, will significantly facilitate experimentation in plant cell biology which often requires all or most of these capabilities.

Materials and methods

Construction of pSAT vectors

Standard DNA amplification and cloning methods were used to construct the basic pSAT vectors. Briefly, the tandem cauliflower mosaic virus (CaMV) 35S promoter, the tobacco etch virus (TEV) leader and the CaMV 35S terminator from pRTL2-GUS (Restrepo *et al*., 1990) were used as regulatory elements for gene expression from all basic pSAT vectors, and open reading frames of EGFP, EYFP, ECFP, DsRed2 (Clontech) and Citrine (Griesbeck *et al*., 2001) were used as N- and C-terminal AFP tags. All plant expression cassettes were cloned into the *Age*I–*Not*I sites of pAUX3133 (Goderis *et al*., 2002), producing pSAT6-EGFP-C1, pSAT6-EYFP-C1, pSAT6-ECFP-C1, pSAT6-Citrine-C1, pSAT6-DsRed2-C1, pSAT6-EGFP-N1, pSAT6-EYFP-N1, pSAT6-ECFP-N1, pSAT6-Citrine-N1, pSAT6-DsRed2-N1, and pSAT6-MCS (GenBank accession numbers AY818377, AY818380, AY818374, AY819771, AY818375, AY818382, AY818378, AY818381, AY818369, AY818373 and AY818383, respectively). The EGFP-C1 expression cassette was also transferred into the *Age*I–*Not*I sites of pAUX3166, pAUX3130, pAUX3169, pAUX3131, pAUX3132, and pAUX3167 (Goderis *et al*., 2002), producing pSAT1-EGFP-C1, pSAT2-EGFP-C1, pSAT3-EGFP-C1, pSAT4-EGFP-C1, pSAT5-EGFP-C1 and pSAT7-EGFP- C1, respectively, with the corresponding GenBank accession numbers AY818363, AY818365, AY818366, AY818367, AY818368, and AY818384. Several test proteins were cloned into the MCSs of pSAT vectors. Briefly, the *Arabidopsis VIP1* (Tzfira *et al*., 2001), *CHS* (Pelletier andShirley, 1996) and *TUA2* (from pU12436, Arabidopsis Biological Resource Center, ARBC) and the cucumber *chrC* (Vishnevetsky *et al*., 1996) ORFs were used to generate pSAT6-EGFP-C1-VIP1, pSAT6-EYFP-C1-CHS, pSAT6-DsRed2-C1-TUA2 and pSAT6-EGFP-N1-cucChrC, respectively. The complete list of available vectors and additional instructions for vector requests is presented in Supplement 1, and detailed descriptions of the cloning strategies are presented in Supplement 2 of the electronic supplementary material.

Assembly of several pSAT expression cassettes into single plasmids

For coexpression of two autofluorescently tagged proteins from a single plasmid, we first produced pSAT1-ECFP-C1-VIP1 by cloning the VIP1 ORF as a *Sal*I-*BamH*I fragment from pSAT6-EGFP-C1-VIP1 into pSAT1-ECFP-C1. The ECFP-VIP1 expression cassette was then cloned as an *Asc*I fragment of pSAT1-ECFP-C1-VIP1 into pPZP-RCS2 (Goderis *et al*., 2002, producing pPZP-ECFP-VIP1. Next, the EYFP-CHS expression cassette was inserted into pPZP-ECFP-VIP1 as a PI-PspI fragment from pSAT6-EYFP-C1-CHS, producing pPZP-ECFP-VIP1/EYFP-CHS. For expression of three autofluorescently tagged proteins from a single plasmid, we first transferred EYFP-CHS as a NcoI-BamHI fragment from pSAT6-EYFP-C1-CHS into pSAT1-EYFP-C1 to produce pSAT1-EYFP-C1-CHS. Next, the SYNV P protein ORF (Goodin, 2002 #2643; Goodin *et al*., 2001) was cloned as a *Sal*I-*Bam*HI fragment into pSAT4-DsRed2-C1, resulting in pSAT4-DsRed2-C1-P. Finally, the ECFP, EYFP-CHS and DsRed2-P expression cassettes were cloned as PI-*Psp*I, *Asc*I and I-*Sce*I fragments from pSAT6-ECFP-C1, pSAT1-EYFP-C1-CHS and pSAT4-DsRed-C1-P into pPZP-RCS2, producing pRCS2-EYFP-CHS/DsRed2-P/ECFP. In addition, the SYNV N protein ORF was cloned as a *Sal*I-*Bam*HI fragment into pSAT3-MCS, producing pSAT3-N.

Promoter and terminator replacement in pSAT vectors

The nopaline synthase promoter (*nos*P) and terminator (*nos*T) were subsequently cloned into the *Age*I-*Nco*I and *Xba*I-*Not*I sites of pSAT6-EGFP-C1, respectively producing pSAT6-nosPnosT-EGFP-C1. A DNA fragment containing three copies of the Gal4 UAS and the TATA box from the CaMV 35S promoter (Aoyama and Chua, 1997) were into the *Age*I–*Nco*I sites of pSAT6-MCS, producing pSAT6-uasP-MCS and the *uidA* gene was added into the MCS producing pSAT6-uasP-GUS. The mGAL4-VP16 activator was cloned into pSAT6-MCS, producing pSAT6-mGAL4VP16.

A 140-bp portion of the *chrC* promoter (140P, A.V., unpublished) was cloned into the *Age*I–*Nco*I sites of pSAT1-EYFP-C1, producing pSAT1-140P-EYFP-C1 and the VIP1 ORF was added to make pSAT1-140P-EYFP-C1-VIP1. The expression cassettes from pSAT1-140P-EYFP-C1-VIP1 and pSAT6-DsRed2-C1 were cloned into pPZP-RCS2 as *Asc*I and PI-*Psp*I fragments, respectively, producing pRCS2-140P-EYFP-VIP1/DsRed2. The mybys ORF (A.V., unpublished) was cloned into pSAT1-MCS, producing pSAT1-mybys and the expression cassettes from pSAT1-mybys and pSAT6-ECFP-C1 were cloned into pPZP-RCS2 as *Asc*I and PI-*Psp*I fragments, respectively, producing pRCS2-mybys/ECFP.

Construction of pRCS2 binary plasmids with selectable markers for transgenic expression

The plant selectable markers *hpt*, *nptII* and *bar* were first cloned into pSAT vectors, producing pSAT1-*hpt*, pSAT4-*nptII*, and pSAT6-*bar* (GenBank accession numbers AY818364, AY818371 and AY818376, respectively), and, from these vectors, the expression cassettes were mobilized to pPZP-RCS2, resulting in RCS2-*hpt*, RCS2-*nptII*, and RCS2-*bar*.

Conversion of pSAT6 to Gateway destination vectors

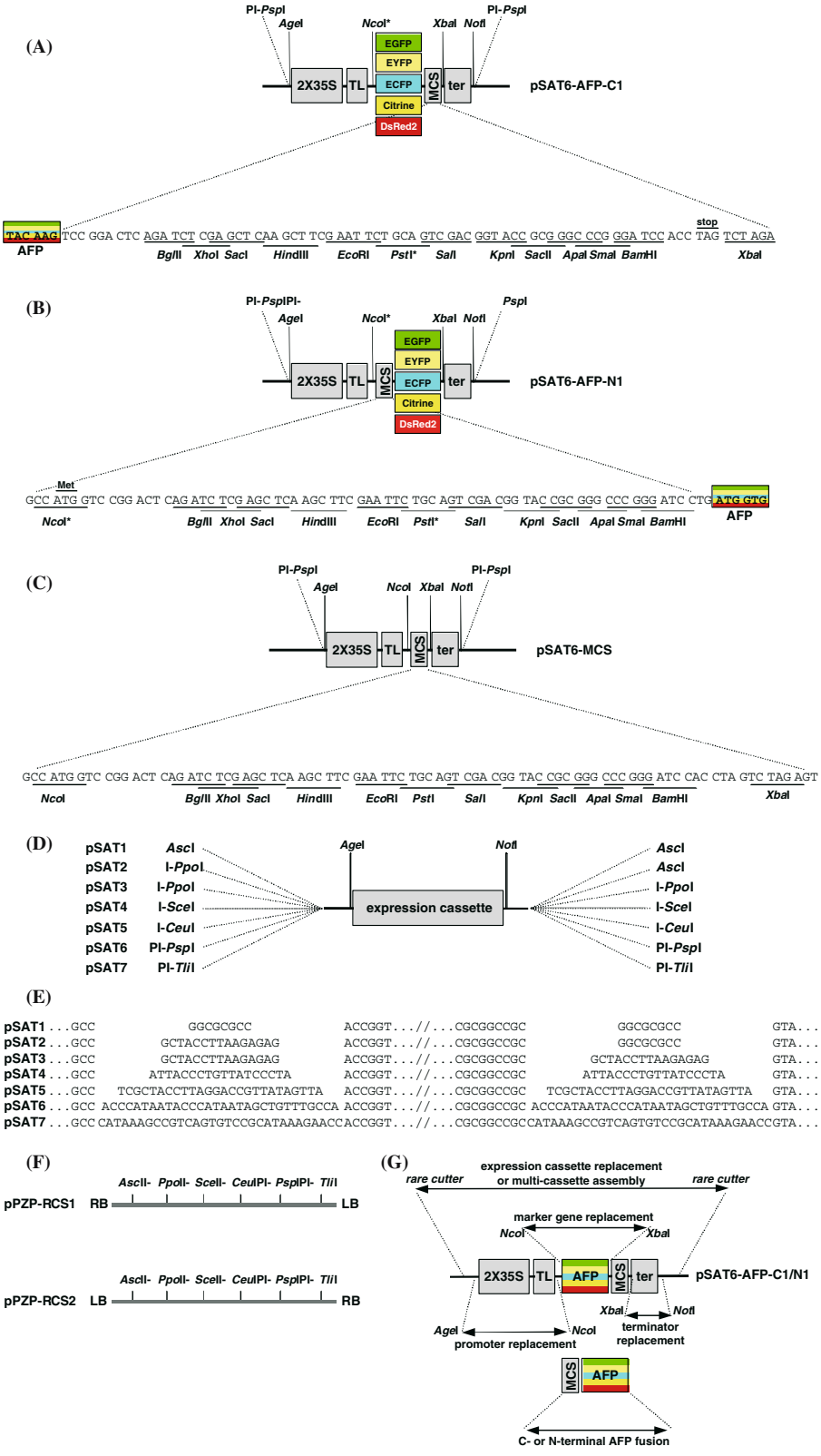
The Gateway conversion cassette (reading frame A, Invitrogen) was used to convert pSAT6-EGFP-N1 to pSAT6-DEST-EGFP-N1, and pSAT6-EGFP-C1 to either pSAT6-DEST-EGFP-C1 or pSAT6-NP-DEST-EGFP-C1 destination vectors, and the donor vector pDONR207 (Invitrogen) was used to produce pDONR-nosP. Detailed descriptions of the plasmid construction strategies and recombination reactions between pSAT6-NP-DEST-EGFP-C1 and pDONR-nosP are available electronically in Supplement 2.

Results and discussion

Design of a modular set of plasmids for expression of AFP tags in plants

We devised a series of ten different satellite (pSAT) vectors for easy construction of C- and N-terminal fusions of five different autofluorescent tags to the N-terminus or the C-terminus, respectively, of a protein of interest. In addition, we constructed a single basic pSAT plasmid for expression of untagged genes. The key features of these plasmids, as exemplified by pSAT6-EGFP-C1, pSAT6-EGFP-N1 and pSAT6-MCS, respectively, are illustrated in Figure 1. The expression of tagged and free proteins is driven by the tandem constitutive promoter of the CaMV 35S RNA; we selected this promoter for its reliable activity in a wide range of plant species and tissues (Hull *et al.*, 2002). To further enhance translation efficiency, the TEV leader (TL) sequence (Restrepo *et al.*, 1990) was inserted downstream of the CaMV 35S promoter. For green, yellow and cyan autofluorescent tags, we chose the enhanced versions of GFP (EGFP) and its yellow (EYFP) and cyan (ECFP) variants (Clontech), which have been codon-optimized for high autofluorescence levels in mammalian and plant cells (Heim *et al.*, 1995; Heim and Tsien, 1996; Yang *et al.*, 1996). As another yellow autofluorescent tag with enhanced photo-stability and tolerance for pH and anions, such as chloride, we used Citrine-YFP (Griesbeck *et al.*, 2001). Finally, as a red autofluorescent tag, we used DsRed2 (Clontech), a mutated version of

Figure 1. Structural features of pSAT series of vectors. The plasmids produce in-frame fusions of the protein of interest to the C-terminus (A) or N-terminus (B) of the AFP tags EGFP, EYFP, ECFP, Citrine-YFP, and DsRed2, or allow expression of free, untagged proteins of interest (C). Expression cassettes are inserted as *Age*I-*Not*I fragments into seven different pSAT plasmids, in which the expression cassettes are flanked with seven unique combinations of rare cutting nuclease recognition sites (D). The complete recognition sequences for these rare cutting nucleases in each vector of the pSAT series are shown (E). Using these rare cutting nucleases, up to seven expression cassettes can be transferred from the pSAT vectors into the T-DNA region of the pPZP-RCS1 and pPZP-RCS2 binary plasmids (F). Individual pSAT vectors are built to produce either C- or N-terminal fusions to each of the AFP tags and can be modified to replace their promoter, AFP marker gene, and/or terminator sequences (G). Restriction endonuclease recognition sites that are not unique in all plasmids are indicated by asterisks; specifically, *Pst*I and *Nco*I recognition sites are also found within the ORFs of EYFP/Citrine-YFP and DsRed2, respectively. Open reading frames for all AFP tags as well as translation initiation and stop codons are indicated. AFP sequences are indicated by colored boxes. 2X35S, tandem CaMV 35S promoter; TL, TEV leader; MCS, multiple cloning site; ter, CaMV 35S transcriptional terminator; C1 and N1 vectors produce fusions to the C- and N-termini, respectively, of the AFP tag.


(A)
PI-PspI
AgeI
NcoI*
XbaI
NotI
PI-PspI
EGFP
EYFP
ECFP
Citrine
DsRed2
2X35S
TL
MCS
ter
pSAT6-AFP-C1
stop
TAC AAG TCC GGA CTC AGA TCT CGA GCT CAA GCT TCG AAT TCT GCA GTC GAC GGT ACC GCG GGC CCG GGA TCC ACC TAG TCT AGA
AFP
BglII XhoI SacI HindIII EcoRI PstI* SalI KpnI SacII ApaI SmaI BamHI XbaI
(B)
PI-PspIPI-
AgeI
NcoI*
XbaI
NotI
PspI
pSAT6-AFP-N1
Met
GCC ATG GTC CGG ACT CAG ATC TCG AGC TCA AGC TTC GAA TTC TGC AGT CGA CGG TAC CGC GGG CCC GGG ATC CTG ATG GTG
NcoI*
AFP
(C)
pSAT6-MCS
GCC ATG GTC CGG ACT CAG ATC TCG AGC TCA AGC TTC GAA TTC TGC AGT CGA CGG TAC CGC GGG CCC GGG ATC CAC CTA GTC TAG AGT
NcoI
XbaI
(D)
pSAT1 AscI
pSAT2 I-PpoI
pSAT3 I-PpoI
pSAT4 I-SceI
pSAT5 I-CeuI
pSAT6 PI-PspI
pSAT7 PI-TliI
expression cassette
AscI
I-PpoI
I-SceI
I-CeuI
PI-PspI
PI-TliI
(E)
pSAT1 . . . GCC GGCGCGCC ACCGGT . . . // . . . CGCGGCCGC GGCGCGCC GTA . . .
pSAT2 . . . GCC GCTACCTTAAGAGAG ACCGGT . . . // . . . CGCGGCCGC GGCGCGCC GTA . . .
pSAT3 . . . GCC GCTACCTTAAGAGAG ACCGGT . . . // . . . CGCGGCCGC GCTACCTTAAGAGAG GTA . . .
pSAT4 . . . GCC ATTACCCTGTTATCCCTA ACCGGT . . . // . . . CGCGGCCGC ATTACCCTGTTATCCCTA GTA . . .
pSAT5 . . . GCC TCGCTACCTTAGGACCGTTATAGTTA ACCGGT . . . // . . . CGCGGCCGC TCGCTACCTTAGGACCGTTATAGTTA GTA . . .
pSAT6 . . . GCC ACCCATAATACCCATAATAGCTGTTTGCCA ACCGGT . . . // . . . CGCGGCCGC ACCCATAATACCCATAATAGCTGTTTGCCA GTA . . .
pSAT7 . . . GCC CATAAAGCCGTCAGTGTCCGCATAAAGAACC ACCGGT . . . // . . . CGCGGCCGC CATAAAGCCGTCAGTGTCCGCATAAAGAACC GTA . . .
(F)
AscII- PpoII- SceII- CeuIPI- PspIPI- TliI
pPZP-RCS1 RB LB
AscII- PpoII- SceII- CeuIPI- PspIPI- TliI
pPZP-RCS2 LB RB
(G)
rare cutter
expression cassette replacement
or multi-cassette assembly
rare cutter
marker gene replacement
NcoI
XbaI
2X35S
TL
AFP
MCS
ter
pSAT6-AFP-C1/N1
XbaI
NotI
terminator
replacement
AgeI
NcoI
promoter replacement
MCS
AFP
C- or N-terminal AFP fusion

DsRed with reduced propensity to aggregate (Baird *et al*., 2000). At the end of the entire expression cassette, we placed the CaMV 35S RNA transcription termination signal.

For easy and versatile fusion of genes of interest with the autofluorescent tags, we constructed an MCS with 13 unique restriction endonuclease recognition sites (12 for EYFP, Citrine-YFP, and DsRed2, due to the presence of internal *Pst*I and *Nco*I recognition sites in the EYFP/Citrine-YFP and DsRed2 open reading frames (ORFs), respectively) (Figure 1A, B). Importantly, this MCS was engineered to maintain the same reading frame in all pSAT vectors, allowing simple, one-step transfer of a target protein sequence between all five autofluorescent tags. In addition, all expression cassettes carried a translation initiation codon (ATG) or a stop codon (TAG) before or after MCS (as illustrated in Figure 1A, B), allowing expression of unfused tags for use in control experiments.

We mounted each of these eleven expression cassettes onto the pAUX3133 plasmid (Goderis *et al*., 2002), that carries a pUC high-copy-number origin of replication and an ampicillin resistance gene for propagation and selection in *E. coli*. This construction step produced a pSAT6 version of our expression vectors, i.e., pSAT6-MCS, pSAT6-EGFP-C1, pSAT6-EYFP-C1, pSAT6-ECFP-C1, pSAT6-Citrine-C1, pSAT6-DsRed2-C1, pSAT6-EGFP-N1, pSAT6-EYFP-N1, pSAT6-ECFP-N1, pSAT6-Citrine-N1, and pSAT6-DsRed2-N1, in which C1 and N1 appellations designate vectors generating fusions to the C- and N-termini of the AFP tags. In pSAT6, the entire expression cassette is flanked by the PI-*Psp*I rare cutting intron-encoded nuclease recognition site (Figure 1D, E), which allows its one-step sub-cloning into pPZP-RCS1 or pPZP-RCS2 *Agrobacterium* binary vectors, containing between their T-DNA borders a MCS with a set of recognition sites for six rare-cutting nucleases *Asc*I, I-*Ppo*I, I-*Sce*I, I-*Ceu*I, PI-*Psp*I and PI-*Tli*I (Figure 1F; Goderis *et al*., 2002).

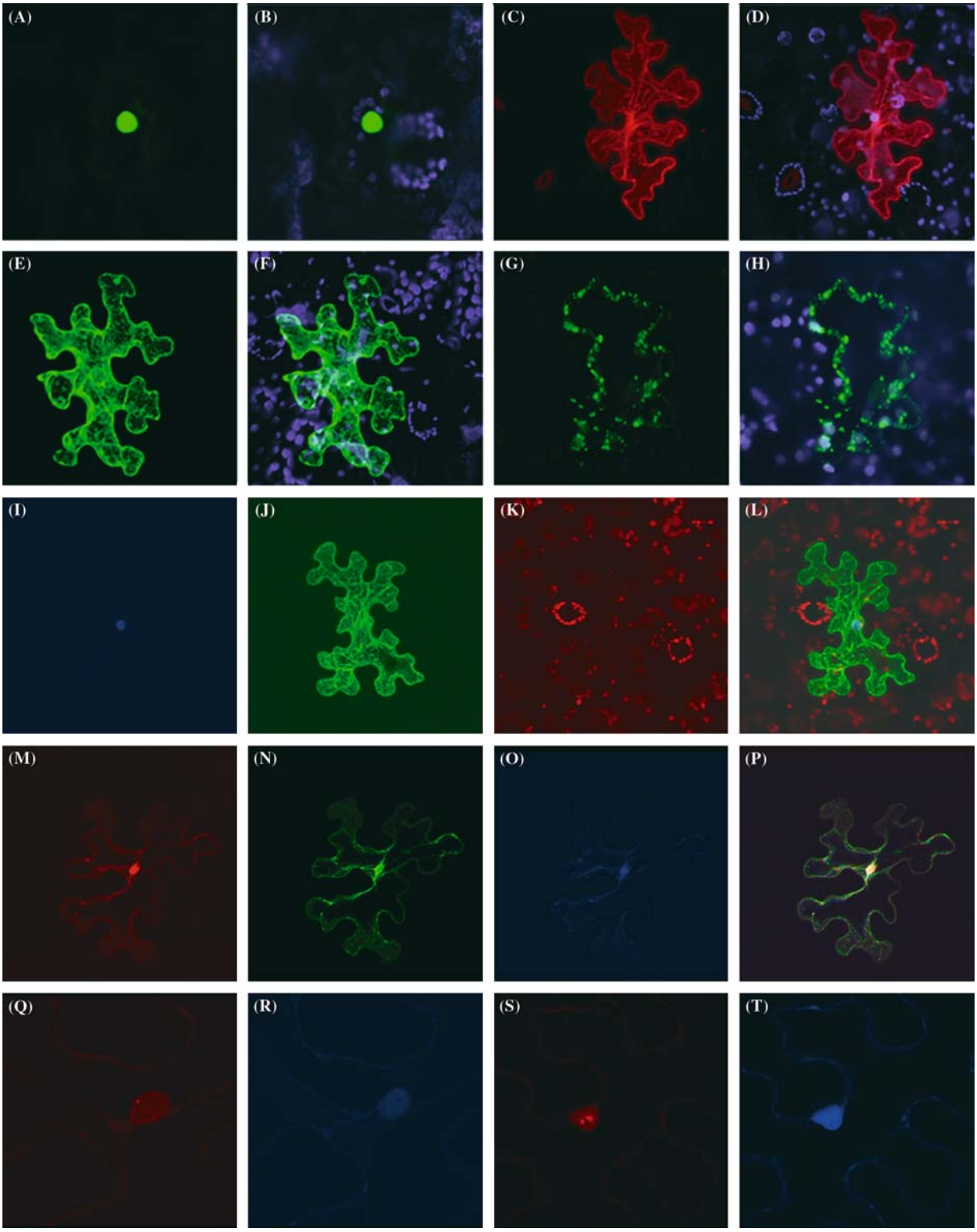
The complete expression cassette can be removed from each of the pSAT6 plasmids as a single *Age*I-*Not*I fragment and transferred to seven pAUX vectors (Goderis *et al*., 2002), producing seven pSAT versions of the same expression cassette. In the resulting series of pSAT constructs, expression cassettes are flanked by *Asc*I (pSAT1), *Asc*I and I-*Ppo*I (pSAT2), I-*Ppo*I (pSAT3), I-*Sce*I (pSAT4), I-*Ceu*I (pSAT5), PI-*Psp*I (pSAT6) and PI-*Tli*I (pSAT7) (Figure 1D, E). Several different expression cassettes flanked by different rare-cutter sites can then be transferred from the pSAT plasmids into the corresponding sites of the pPZP-RCS1 or pPZP-RCS2 plasmids, resulting in a single vector that expresses multiple genes, each fused to a different autofluorescent tag (see below).

Thus, we produced a series of pSAT vectors with seven major utility and versatility features summarized in Figure 1G: (i) gene fusions to five different autofluorescent tags, (ii) a choice of C- and N-terminal fusion to each tag, (iii) single cloning strategy for all tags, (iv) single reading frame for all tags, (v) the ability to assemble multiple expression cassettes in a single binary vector for simultaneous expression of multiple genes, each tagged with a different autofluorescent marker, (vi) one-step exchange of the expression cassettes between different pSAT vectors, (vii) one-step replacement of promoter and/or terminator sequences in each of the expression cassettes.

Autofluorescent gene tagging in pSAT6 plasmid series: proof of concept

To test the functionality of pSAT plasmids in plant cells, we used them to tag several plant proteins

Figure 2. Targeting of fluorescently tagged plant proteins to diverse sub-cellular compartments and structures following expression from pSAT expression cassettes. (A, B) EGFP-VIP1 expressed from pSAT6-EGFP-C1-VIP1 targets to the cell nucleus. (C, D) DsRed2-TUA2 expressed from pSAT6-DsRed2-C1-TUA2 targets to microtubules. (E, F) EYFP-CHS expressed from pSAT6-EYFP-C1-CHS targets to the rough ER. (G, H) EGFP-cucChrC expressed from pSAT6-EGFP-N1-cucChrC targets to proplastids and chloroplasts. (I-L) ECFP-VIP1 and EYFP-CHS expressed from pRCS2-ECFP-VIP1/EYFP-CHS target to the cell nucleus and rough ER, respectively. (M-P) DsRed2-P, EYFP-CHS and free ECFP expressed from pRCS2-EYFP-CHS/DsRed-P/ECFP. (Q, R) Mostly diffuse nuclear localization of DsRed2-P and free ECFP expressed from pRCS2-EYFP-CHS/DsRed-P/ECFP. (S, T) Re-localization of DsRed2-P into sub-nuclear structures when expressed from pRCS2-EYFP-CHS/DsRedP/ECFP in the presence of the SYNV N protein expressed from pSAT3-N. Panel a shows EGFP signal in green; panels C, M, Q, and s show DsRed2 signal in red; panels E, J, and F show EYFP signal in green; panels I, O, R, and T, show ECFP signal in blue. Plastid autofluorescence is in purple in panels A–H and in red in panels K and L. Panels B, D, F, H, L, and P represent merged signals of the corresponding AFP and plastid autofluorescence. Images in panels A, B, G, H, and Q–T are single confocal sections, and panels C, D, E, F and I–P are projections of several confocal sections.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(T)

known to localize in diverse sub-cellular compartments and structures, such as the nucleus, microtubules, the endoplasmic reticulum (ER), and plastids. The tagged proteins were transiently expressed in epidermal cells of tobacco leaves following biolistic delivery of their encoding pSAT constructs, and the sub-cellular localization of the fluorescent protein fusions was determined using confocal laser scanning microscopy as previously described (Lacroix *et al*., 2005). Figure 2 shows that the nuclear *Arabidopsis* protein VIP1 (Tzfira *et al*., 2001, 2002) tagged with EGFP indeed accumulated in the nucleus of tobacco epidermal cells (Figure 2A, B), while DsRed2-tagged *Arabidopsis* alpha-2,4 tubulin encoded by the *TUA2* gene formed a fine intracellular network characteristic of cytoskeletal proteins (Ludin andMatus, 1998; Erhardt *et al*., 2002) (Figure 2C, D). We demonstrated ER localization (Figure 2E, F) using a fusion of EYFP to chalcone synthase (CHS) (Pelletier and Shirley, 1996), an enzyme in the flavonoid biosynthesis that is known to reside on the cytoplasmic face of the rough ER (Hrazdina *et al*., 1987; Saslowsky and Winkel-Shirley, 2001). EYFP-CHS was also found around the nuclear envelope (Figure 2E, F), a localization pattern previously observed for other ER-associated fluorescently tagged proteins in plant cells (Escobar *et al*., 2003; Brandizzi *et al*., 2004) and in *C. elegans* neurons (Rolls *et al*., 2002). Finally, proplastid and plastid targeting was demonstrated using a fusion between the chromoplast-specific carotenoid-associated protein ChrC from cucumber (Vishnevetsky *et al*., 1996, 1999) and EGFP (Figure 2G, H).

Plasmid modularity: gene replacement and multiple gene expression

To demonstrate the suitability of the pSAT vectors for easy transfer of target genes between different AFP tags and for expression of multiple genes from a single vector, we first mounted two expression cassettes onto a single plasmid pPZP-RCS2. One cassette expressed VIP1 tagged with CFP from the pSAT1-ECFP-C1-VIP1 vector, and the other cassette expressed EYFP-tagged CHS from pSAT6-EYFP-C1-CHS. Microbombardment and expression of the resultant vector, pRCS2-ECFP-VIP1/EYFP-CHS, in leaves resulted in nuclear accumulation of ECFP-VIP1 (Figure 2I) and in the association of EYFP-CHS with the rough ER (Figure 2J); both AFP tags were also clearly distinguishable in the merged images even against the autofluorescent background of chloroplasts (Figure 2K, L). Next, we designed an experiment not only to monitor expression of multiple proteins from a single vector, but also to detect an induced re-localization of a protein within plant cells. To this end, we mounted three expression cassettes, onto a single pPZP-RCS2 plasmid. The first cassette expressed EYFP-CHS from pSAT1-EYFP-C1-CHS, the second cassette expressed the P protein of *Sonchus yellow net rhabdovirus* (SYNV) (Goodin *et al*., 2001, 2002) tagged with DsRed2 from pSAT4-DsRed2-C1-P, and the third cassette expressed free ECFP from pSAT6-ECFP-C1. The resulting vector, pRCS2-EYFP-CHS/DsRed2-P/ECFP, was microbombarded into tobacco leaves. Consistent with previous observations (Hrazdina *et al*., 1987; Goodin *et al*., 2001, 2002; Saslowsky and Winkel-Shirley, 2001), DsRed2-P as well as free CFP were found both within the cell nucleus and the cytoplasm (Figure 2M, N) while EYFP-CHS decorated the rough ER and the nuclear envelope (Figure 2N, E). Closer examination of the cell nucleus at higher magnification revealed fine differences between DsRed2-P and CFP; while the latter was evenly distributed inside the nucleus (Figure 2R), the former showed some aggregation at the nuclear envelope (Figure 2Q). We then co-bombarded pPZP-EYFP-CHS/DsRed2-P/ECFP with pSAT3-N which expressed free SYNV N protein known to interact with the P protein and to translocate it into sub-nuclear compartments, presumably the nucleolus (Goodin *et al*., 2001, 2002). Indeed, Figure 2S shows that, in these co-transformed cells, DsRed2-P predominantly accumulated in distinct sub-nuclear structures. This re-localization was specific because it was not observed with CFP expressed in the same cell (Figure 2T). These results illustrate the suitability of the pSAT system for multiple gene expression as well as for functional analysis of protein–protein interactions *in planta*.

Plasmid modularity: promoter and terminator replacement

While CaMV 35S promoter and terminator sequences are perhaps the most widely used regulatory elements for gene expression in plant cells

(Hull *et al.*, 2002), truly useful and versatile expression vectors must have an ability to easily substitute these standard regulatory sequences with those of interest for individual researchers, e.g., inducible or tissue-specific promoters or native regulatory elements of the tagged gene itself. The pSAT series of plant expression vectors allows such capability; specifically, the tandem CaMV 35S promoter with its adjacent TL sequence and the CaMV 35S terminator can be removed and replaced by double-digestion with either *Age*I and *Nco*I or *Xba*I and *Not*I, respectively (Figure 1g). However, caution should be taken when replacing the promoter in DsRed2-containing plasmids due to the presence of an additional *Nco*I site within the DsRed2 coding sequence. The resulting expression cassettes with new regulatory elements can be mobilized between different pSAT vectors as single *Age*I-*Not*I fragments (Figure 1D).

We first demonstrated these capabilities of the pSAT constructs by replacing the CaMV 35S promoter and terminator sequences of pSAT6-EGFP-C1 with those of the nopaline synthase (*nos*) gene, i.e., *nos* promoter (*nos*P) and *nos* terminator (*nos*T), resulting in the pSAT6-nosPnosT-EGFP-C1 construct. The entire expression cassette was then removed and mobilized to pSAT5 as an *Age*I-*Not*I fragment, producing pSAT5-nosPnosT-EGFP-C1. Both constructs were functional, producing essentially identical GFP expression patterns following transient expression in tobacco epidermis (data not shown).

Next, we illustrated how pSAT vectors can be used to construct expression vectors for promoter transactivation in plant cells. To this end, we chose a promoter containing three copies of the GAL4 upstream activating sequence (UAS) and its chimeric activator composed of the transcriptional

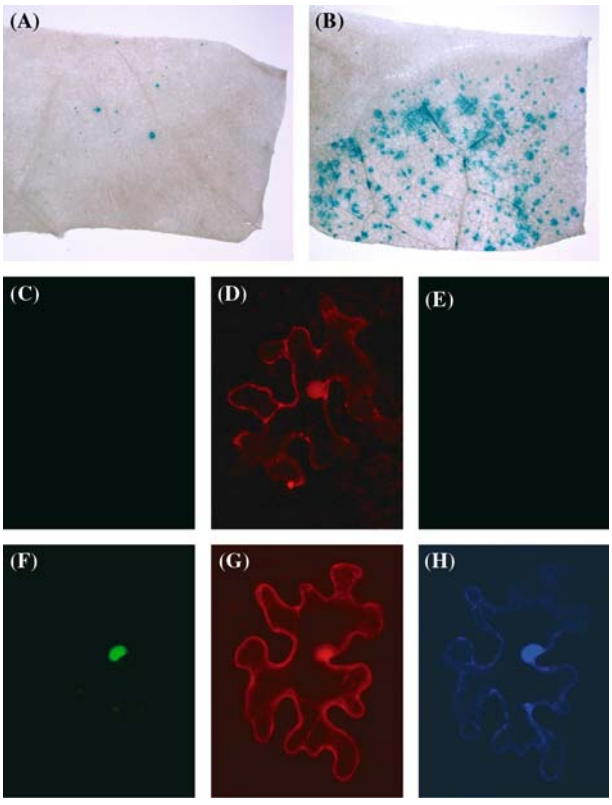

Figure 3. Gene expression from pSAT vectors with replaced promoters. (A) Residual GUS activity in tobacco leaves transformed with pSAT6-uasP-GUS. (B) Strong GUS activity in tobacco leaves co-transformed with pSAT6-uasP-GUS and pSAT6-mGAL4VP16. (C–E) Tobacco cells transformed with pRCS2-140P-EYFP-VIP1/DsRed2 do not express EYFP-VIP1, express DsRed2, and do not show ECFP signal. (F–H) Tobacco cells co-transformed with pRCS2-140P-EYFP-VIP1/DsRed2 and pRCS2-mybys/ECFP express EYFP-VIP1, DsRed2 and ECFP. EYFP signal is in green, DsRed2 signal is in red, and ECFP signal is in blue; plastid autofluorescence was filtered out.

activation domain of the *Herpes simplex* virus protein VP16 fused to the Gal4 DNA binding domain modified for optimal expression in *Arabidopsis* (mGAL4, see www.plantsci.cam.ac.uk/Haseloff/Home.html). We then replaced the CaMV 35S promoter of pSAT6-MCS with the UAS promoter (*uasP*) and inserted the GUS reporter downstream to the new promoter. When biolistically introduced into tobacco epidermis, the resulting pSAT6-uasP-GUS vector was capable of only residual GUS expression, as very few blue spots could be observed following histochemical staining of the entire bombardment area for GUS activity (Figure 3A). In contrast, co-bombardment of pSAT6-uasP-GUS with pSAT6-mGAL4VP16, that expresses the mGAL4-VP16 fusion from the CaMV 35S promoter, resulted in massive expression of the GUS reporter (Figure 3B) and, therefore, in efficient transactivation of the UAS promoter.

Finally, we demonstrated the use pSAT vectors for transactivation experiments using endogenous plant promoter. We focused on the cucumber *chrC* promoter, which normally is active in chromoplastogenic tissues (e.g., cucumber corollas, Vishnevetsky *et al.*, 1999), but a 140-bp fragment of which can be induced in other tissues in several plant species, including *Arabidopsis* and tobacco, by co-expression of a cucumber Myb-like gene, *mybys* (A.V., unpublished). We replaced the 35S promoter of pSAT1-EYFP-C1 with the 140-bp partial *chrC* promoter (140P), and fused VIP1 to the YFP tag, producing pSAT1-140P-EYFP-C1-VIP1. Next, the EYFP-VIP1 expression cassette from pSAT1-140P-EYFP-C1-VIP1 was transferred, together with a cassette expressing free DsRed2 from pSAT6-DsRed2-C1, into pPZP-RCS2, producing the pRCS2-140P-EYFP-VIP1/DsRed2 double expression construct. In parallel, we cloned the *mybys* ORF under the 35S promoter of pSAT1-MCS to produce pSAT1-mybys and mounted its expression cassette, together with a free ECFP expression cassette from pSAT6-ECFP-C1, onto pPZP-RCS2, resulting in the pRCS2-mybys/ECFP double-expression construct. Thus, pRCS2-140P-EYFP-VIP1/DsRed2 expresses a reporter gene (EYFP-VIP1) to discover the tested promoter activity and free fluorescent marker (DsRed2) to identify the cells that received pRCS2-140P-EYFP-VIP1/DsRed2 while pRCS2-mybys/ECFP expresses the transactivator (mybys) of the tested promoter and a different fluorescent marker (ECFP) to identify the cells that received pRCS2-mybys/ECFP. When pRCS2-140P-EYFP-VIP1/DsRed2 was bombarded alone into tobacco leaves, it failed to produce YFP fluorescence in DsRed2-expressing cells (Figure 3c-e), indicating that the 140-bp partial *chrC* promoter is inactive in these target tissues. Co-bombardment of pRCS2-140P-EYFP-VIP1/DsRed2 and pRCS2-mybys/ECFP resulted in activation of the 140-bp partial *chrC* promoter as indicated by the appearance of the EYFP-VIP1 fusion protein which accumulated in the nuclei of the DsRed2/ECFP-expressing cells (Figure 3F–H).

Agrobacterium-mediated delivery of pSAT expression cassettes for stable expression of multiple genes in transgenic plants

It would be useful if, in addition to their application for transient gene expression, the pSAT vectors could be expressed stably, in transgenic plants, following their transfer to the pPZP-RCS2 binary plasmid (Goderis *et al.*, 2002). To this end, we modified pPZP-RCS2 to express different transformation selection markers. First, we produced a set of three expression cassettes, pSAT1-*hpt*, pSAT4-*nptII* and pSAT6-*bar,* carrying genes encoding the resistance to hygromycin, kanamycin and phosphinothricin, respectively, and then transferred them into pPZP-RCS2 to produce pRCS2-*hpt*, pRCS2-*nptII* and pRCS2-*bar* (Figure 4A). To illustrate the use of these constructs in conjunction with a pSAT expression cassette for stable gene expression, we constructed a binary plasmid pRCS2-*nptII*-DsRed2/EGFP with three expression cassettes encoding kanamycin resistance (*nptII*), free DsRed2, and free EGFP derived from pSAT4-*nptII*, pSAT1-DsRed2-C1, and pSAT6-EGFP-C1, respectively. This binary vector was introduced into *Agrobacterium* and used to genetically transform tobacco leaf disks (Horsch *et al.*, 1985). Following regeneration of kanamycin-resistant plants, the constitutive expression of DsRed2 and EGFP was observed in diverse tissues, such as guard cells, leaf epidermis, and trichomes, of these transgenic plants (data not shown), demonstrating the effectiveness of this system for stable expression of multiple genes in transgenic plants.

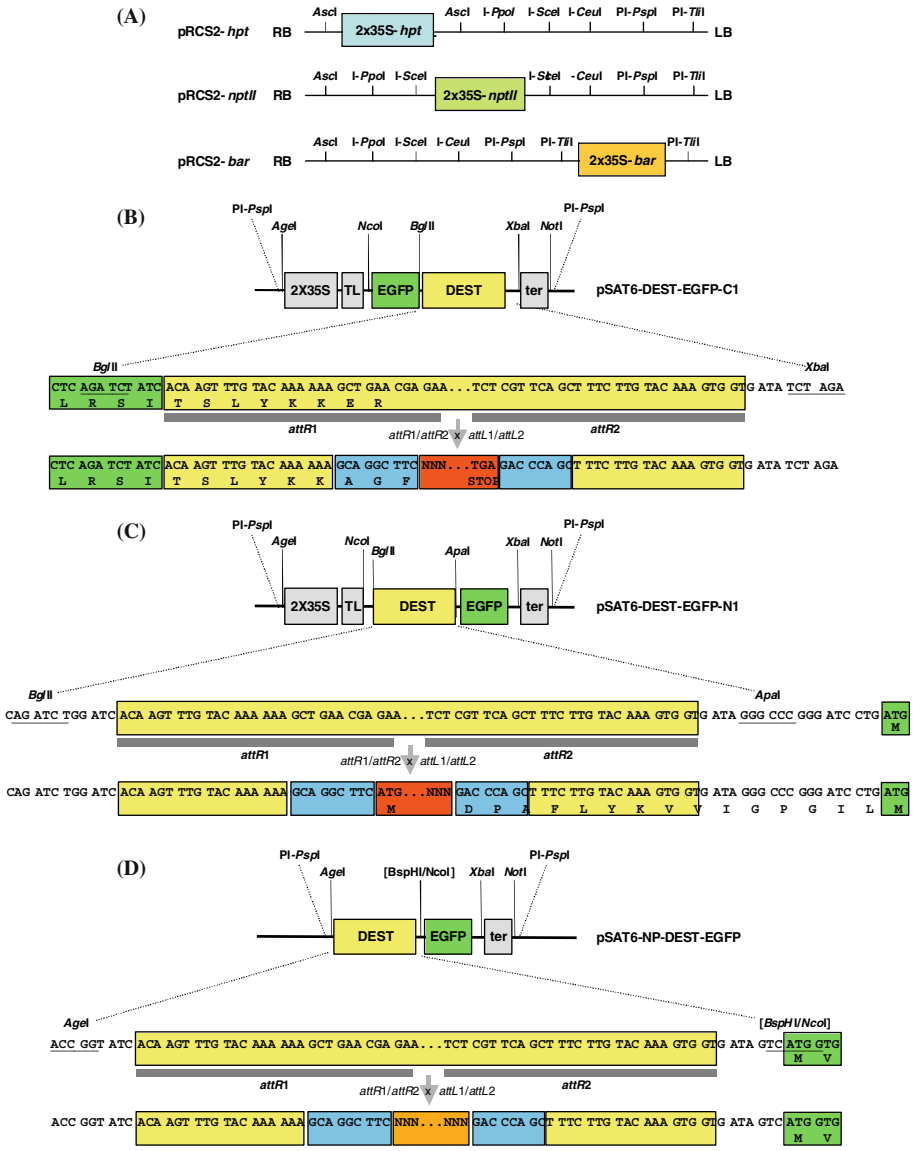

Figure 4. Structural features of the pRCS2 binary plasmids and the basic pSAT6 Gateway destination vectors. (A) pRCS2 binary plasmids designed to accept multiple expression cassettes from pSAT vectors and carrying antibiotic resistance genes *hpt*, *nptII*, or *bar* driven by tandem CaMV35S promoters (indicated by blue, green, and orange boxes, respectively) for selection of transgenic plants. (B–C) Gateway destination plasmids that allow recombination of the protein of interest for in-frame fusions to the C-terminus (A) or N-terminus (B) of the EGFP tag or recombination of the promoter of interest upstream of the EGFP reporter (C). Unique restriction endonuclease recognition sites are indicated; brackets indicate the cloning junction which does not reconstitute its parental recognition sites. For each destination vector, recognition sites for the rare cutting nuclease PI-*Psp*I that flank the entire expression cassette are indicated, and sequences of the EGFP fusion junction, before and after the *attL* × *attR* recombination, are shown. Open reading frames for the EGFP tags as well as translation initiation and stop codons for N-terminal and C-terminal tagging, respectively, are indicated. Amino acid sequences are shown in one-letter code. EGFP sequences are indicated by green boxes, Gateway destination cassette sequences are indicated by yellow boxes, the *att*R1 and *att*R2 sites within the destination cassette are indicated by gray bars, the recombined gene or promoter are indicated by red or orange boxes, respectively, and their flanking sequences produced following recombination are indicated by blue boxes.

We adapted the already highly versatile pSAT vectors for high-throughput recombination cloning using the Gateway™ technology (Walhout *et al.*, 2000). We produced three pSAT Gateway destination plasmids, pSAT6-DEST-EGFP-C1, pSAT6-DEST-EGFP-N1 and pSAT6-NP-DEST-EGFP, into which the Gateway conversion cassette was introduced instead of the N- or C-terminal MCS or the CaMV 35S promoter, respectively (Figure 4B–D). The location of the *attR*1 and *attR*2 recombination sites in pSAT6-DEST-EGFP-C1 and pSAT6-DEST-EGFP-N1 was designed to produce in-frame fusions between the gene of interest, flanked by the *attL*1 and *attL*2 sequences and delivered by a Gateway entry vector, and the EGFP tag following the *attL* x *attR* recombination (Figure 4B, C). The location of *attR1* and *attR2* sites in pSAT6-NP-DEST-EGFP was designed to allow recombination cloning of promoters of interest upstream of the EGFP reporter (Figure 4D). This capability was illustrated by the observations that pSAT6-NP-DEST-EGFP failed to produce EGFP fluorescence when biolistically delivered into tobacco cells but that the EGFP signal was observed when the nopaline synthase promoter from the donor plasmid pDONR-nosP was recombined into pSAT6-NP-DEST-EGFP, producing pSAT6-nosP-EGFP and resulting in reconstruction of a functional EGFP expression cassette (data not shown).

In summary, we described the construction and experimental applications of a new family of plant expression plasmids for autofluorescent gene tagging, expression of multiple tagged and untagged genes from a single vector, easy replacement of the tagged genes and their regulatory elements, Gateway recombination cloning, and the capacity for both transient and stable, transgenic gene expression. We hope that these vectors will further facilitate experimentation in plant cell biology which often requires all or most of these capabilities.

Acknowledgments

We thank Dr Jim Haseloff for the gift of pET-15GAL4-VP16UASmGFP5ER, Dr Nam-Hai Chua for the gift of pTA7001, and Dr Brenda Winkel Shirley for the gift of pCHS.CR2. This study was supported by a grant from the Human Frontiers Science Program to T.T. The work in our labs is also supported by grants from US–Israel Binational Research and Development Fund (BARD) to T.T., from the National Institutes of Health, National Science Foundation, U.S. Department of Agriculture, US–Israel Binational Science Foundation (BSF) and BARD to V.C., and BARD and Israel Science Foundation (ISF) to A.V.

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