PLANT TRANSFORMATION TECHNOLOGIES

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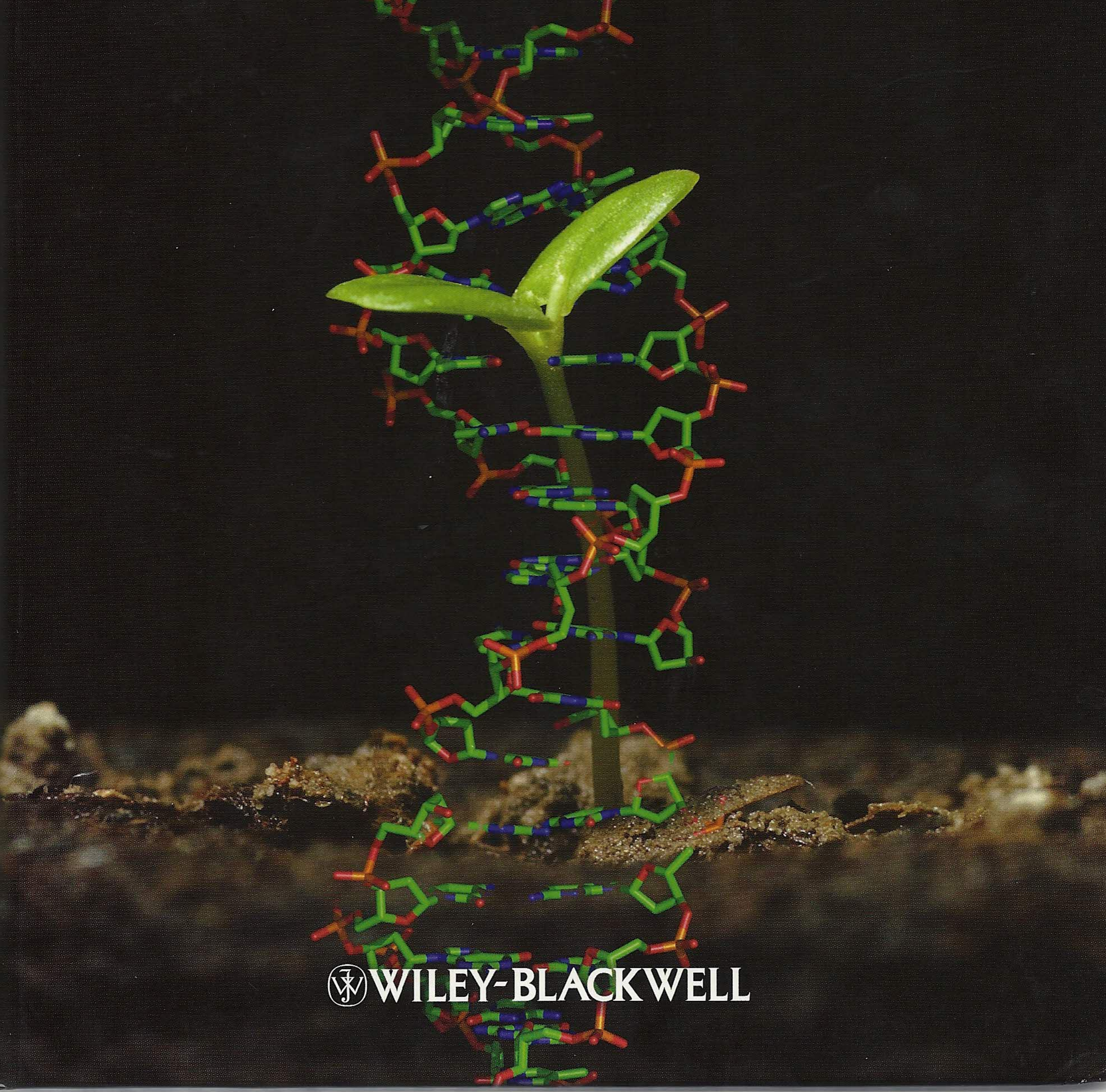

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11 pANIC: A Versatile Set of Gateway-Compatible Vectors for Gene Overexpression and RNAi-Mediated Down-Regulation in Monocots

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Why Make a New Vector Set for Grass Transformation?

The genetic improvement of crops and forward genetics studies of various plant species has grown rapidly in the past few decades. Plant transformation is a valuable tool in these processes, whereby a plasmid vector carrying the transgene of interest is used to facilitate gene transfer into the plant species, using techniques such as particle bombardment or *Agrobacterium*-mediated transformation resulting in stable insertion of the transgene into the chromosomal DNA. In this way, the function of heterologous genes can be elucidated through overexpression analysis, and endogenous gene function can be elucidated by utilizing RNA interference (RNAi)-mediated down-regulation mechanisms.

The BioEnergy Science Center is a US Department of Energy-funded bioenergy center, focused on reverse genetics screens of cell wall biosynthesis genes in stably transgenic hybrid poplars and switchgrass. These screens must be accomplished in a high-throughput manner. Several plasmid vector sets are available for use and described in the scientific literature for a variety of transgenic applications, but none are ideally suitable for high-throughput forward genetics screens in monocots, namely, they lack ease of assembly or flexibility for a variety of transformation methods. A novel vector set is described here to facilitate high-throughput vector construction for production of stable transgenics.

Existing Plant Expression Vectors

Traditional binary vectors, such as pBIN (Bevan 1984), pGA (An *et al.* 1985), pBI (Jefferson *et al.* 1987), pCB (Xiang *et al.* 1999), and newer vectors such as pPZP (Hajdukiewicz *et al.* 1994), and pCAMBIA (http://www.cambia.org/daisy/cambia/585.html) are widely used in dicots and have been improved upon and used as backbones for other vector sets. For instance, the pPZP-RCS2 (Goderis *et al.* 2002), pUGA (Thomson *et al.* 2002) and Gateway destination (Karimi *et al.* 2002) vector series are derivatives of the pPZP vectors, with pPZP-RCS2 and pUGA containing the addition of rare-cutting restriction enzyme sites and Gateway destination vectors containing the addition of the Gateway cassette (*attR1*-Cm^R-*ccdB*-*attR2*, discussed in further details below).

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To facilitate a wide range of needs in plant genetic studies, plasmid vector sets have been constructed with reporter genes for promoter analysis (Jefferson *et al.* 1987; Coutu *et al.* 2007; Nakagawa *et al.* 2007; Nakagawa *et al.* 2008), with reporter fusions for expression and protein localization (Goodin *et al.* 2002; Tzfira *et al.* 2005; Chakrabarty *et al.* 2007), with Gateway sites for rapid cloning of genes (Wesley *et al.* 2001; Karimi *et al.* 2002; Curtis and Grossniklaus 2003; Miki and Shimamoto 2004; Tzfira *et al.* 2005), and with Gateway sites for multiple transgene delivery capabilities (Tzfira *et al.* 2005; Chen *et al.* 2006). Many of these vector sets contain a combinatorial variety of the features described above.

Current Limitations

Currently, multiple binary vector sets are also available for transgene functional analysis by means of overexpression or down-regulation. Most of the published plant vector sets rely on a limited selection of constitutive promoters to drive transgene expression. The cauliflower mosaic virus (CaMV) 35S or double CaMV 35S (2×35S or d35S) promoters can be used for high levels of constitutive expression in a broad range of tissue types, and these promoters are typically implemented for transgene regulation or selectable marker gene expression (Wesley *et al.* 2001; Goodin *et al.* 2002; Curtis and Grossniklaus 2003; Earley *et al.* 2006; Nakagawa *et al.* 2007). The 35S promoter can be used in rice (Battraw and Hall 1990); however, it results in minimal levels of expression in other monocot species (Himmelbach *et al.* 2007; Mazarei *et al.* 2008). The maize ubiquitin promoter and intron (*ZmUbi*1) or the rice actin promoter and intron (*OsAct*1) are most commonly used for heterologous expression of transgenes in monocots (Miki and Shimamoto 2004; Himmelbach *et al.* 2007; Kim *et al.* 2009), but the availability of these promoters is limited in versatile plant expression vector sets.

Features of pANIC

Because of the limited capabilities of vectors for transgene expression in monocots, we have designed and constructed a versatile set of 16 gateway-compatible destination vectors (termed "pANIC", named after the genus of our primary target, *Panicum virgatum*, switchgrass). One reason for creating the vectors is to unify switchgrass transgene expression platforms among groups in our bioenergy center (Table 11.1). Gateway compatibility allows for convenient insertion of any open reading frame (ORF) or other target sequence of interest. Because of the speed and ease of this cloning technology when compared to that of traditional cloning methods in plant transformation vectors, sequences of interest can be screened much faster, resulting in higher throughput and analysis of target genes. The pANIC vectors can be used for (1) transgene overexpression or (2) targeted gene silencing, using double-stranded RNA interference (RNAi). Since both biolistic bombardment and *Agrobacterium*-mediated transformation procedures are routinely used for monocots, the pANIC vector set includes vectors that can be utilized for both applications. All vectors contain three basic elements: (1) a Gateway compatible cassette for overexpression or down-regulation of the target gene, (2) a plant selectable marker cassette for conferring resistance (*bar* or *hph*) to the transformed plant, and (3) a visual reporter gene cassette (GUSPlus or pporRFP) for optimization of the transformation method, visual tracking, and rapid identification of transgenic plants. The pANIC vector set allows for high-throughput screening of transgenes in monocot plant species (Figure 11.1).

Table 11.1. Details of the pANIC vector set.

Name of vector	OE or RNAi	Promoter for gateway cassette	Selection	Reporter gene	Biolistic or binary backbone
pANIC 5A	OE	ZmUbi1	*hph*	pporRFP	Biolistic
pANIC 5B	OE	ZmUbi1	*hph*	GUSPlus	Biolistic
pANIC 5D	OE	ZmUbi1	*bar*	pporRFP	Biolistic
pANIC 5E	OE	ZmUbi1	*bar*	GUSPlus	Biolistic
pANIC 6A	OE	ZmUbi1	*hph*	pporRFP	Binary
pANIC 6B	OE	ZmUbi1	*hph*	GUSPlus	Binary
pANIC 6D	OE	ZmUbi1	*bar*	pporRFP	Binary
pANIC 6E	OE	ZmUbi1	*bar*	GUSPlus	Binary
pANIC 7A	RNAi	ZmUbi1	*hph*	pporRFP	Biolistic
pANIC 7B	RNAi	ZmUbi1	*hph*	GUSPlus	Biolistic
pANIC 7D	RNAi	ZmUbi1	*bar*	pporRFP	Biolistic
pANIC 7E	RNAi	ZmUbi1	*bar*	GUSPlus	Biolistic
pANIC 8A	RNAi	ZmUbi1	*hph*	pporRFP	Binary
pANIC 8B	RNAi	ZmUbi1	*hph*	GUSPlus	Binary
pANIC 8D	RNAi	ZmUbi1	*bar*	pporRFP	Binary
pANIC 8E	RNAi	ZmUbi1	*bar*	GUSPlus	Binary

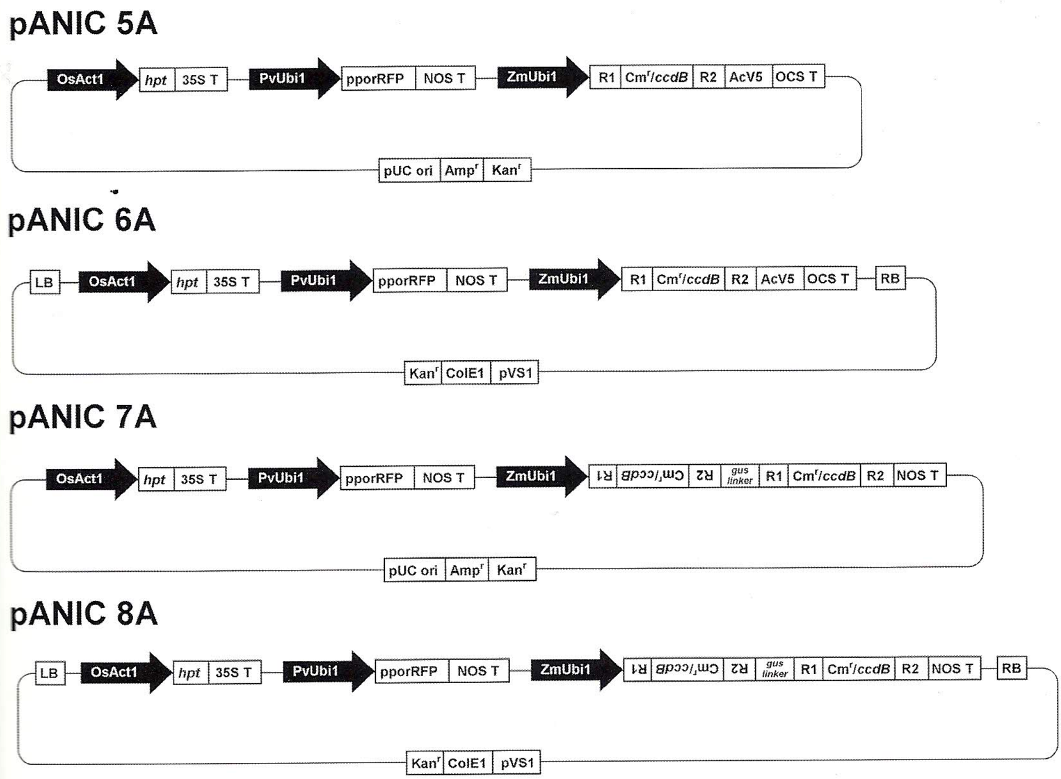

Figure 11.1. Representative maps of the pANIC vector set.

Replication Origins and Bacterial Selection

The vectors for biolistic bombardment (pANIC 5x and 7x series) contain the pCR4 backbone (Invitrogen, Carlsbad, CA). This backbone contains the pUC origin of replication for *Esherichia coli*, along with the ampicillin (*bla*) and kanamycin resistance genes. For binary vectors (pANIC 6x and 8x series), we used the pPZP201BK backbone (Covert *et al.* 2001). This vector contains the bacterial kanamycin resistance gene (*nptI*). The ColE1 origin of replication results in high copy number in *E. coli* and the broad host range pVS1 origin allows for low copy number replication in *Agrobacterium*. The pBR322 *bom* site is present for conjugational transfer, and the presence of the *rep* and *sta* regions from pVS1 make these binary vectors stable in *Agrobacterium* even in the absence of selection pressure (Hajdukiewicz *et al.* 1994).

Gateway Cloning for Overexpression and RNAi

Gateway cloning is a powerful molecular biology tool that takes advantage of the bacteriophage lambda site-specific recombination system, facilitating the exchange of DNA fragments from an "entry" vector to a "destination" vector in an efficient and highly dependable manner (Hartley *et al.* 2000). The pANIC vectors constitute a set of "destination" vectors, containing the *attR1* and *attR2* recombination sites flanking (1) the chloramphenicol resistance gene (Cm^R) and (2) an additional selectable marker gene (*ccdB*) that kills *E. coli* cells by the induction of gyrase-mediated double-stranded DNA breakage. After recombination with an entry vector containing the DNA sequence of interest, the selection pressure of the *ccdB* gene, coupled with selection for an antibiotic resistance marker gene, drastically decreases the background colonies and increases the efficiency of detecting a positive colony containing the DNA sequence of interest in the recombined expression vector.

Regulatory Elements for Expression of Genes of Interest

In the overexpression vectors (pANIC 5x and pANIC 6x series), expression of the target sequence is under constitutive transcriptional regulation of the maize ubiquitin-1 promoter containing an intron (*ZmUbi*1) (Christensen *et al.* 1992). The *ZmUbi*1 promoter is widely used in monocot species for high levels of constitutive expression, including expression in maize (Christensen *et al.* 1992), rice (Toki *et al.* 1992), sugarcane (Wei *et al.* 2003), wheat (Vasil and Vasil 2006), turfgrass (Agharkar *et al.* 2007), and switchgrass (Mazarei *et al.* 2008). For subsequent characterization studies of the transformed plants, immediately downstream of the *attR2* site is a sequence for the epitope tag AcV5. This C-terminal epitope tag can be used for protein purification and characterization (Earley *et al.* 2006). The octopine synthase (OCS) terminator is used for transcriptional termination. In the RNAi vectors (pANIC 7x and pANIC 8x series), we utilized the well-established RNAi cassette from the pANDA vector set (Miki and Shimamoto 2004), containing the *attR1*-Cm^R-*ccdB*-*attR2* region followed by an inverted repeat of itself, resulting in a hairpin loop of the target sequence after recombination and transcription. Transcriptional regulation of the RNAi target sequence is driven by the *ZmUbi*1 promoter, and the *A. tumefaciens* nopaline synthase (NOS) terminator is used for termination.

Plant Selection Cassettes

The only selectable marker gene described for stable switchgrass transformation in the literature is the bialaphos acetyltransferase (*bar*) gene. The *bar* gene confers resistance to bialaphos, a structural analog of glutamate that inhibits glutamate synthase (Tachibana *et al.* 1986). Richards *et al.* used the *bar* gene driven by the *ZmUbi*1 promoter for transgenic switchgrass selection (Richards *et al.* 2001), and Somleva (2007) and Somleva *et al.* (2002, 2008) have consistently used this same selection cassette (*ZmUbi*1-*bar*). The hygromycin phosphotransferase (*hph*) gene has been used extensively in dicot and monocot vectors for selection of stably transformed plants (Rothstein *et al.* 1987; Dekeyser *et al.* 1989; Walters *et al.* 1992; Zhu *et al.* 1993; Draper *et al.* 2001; Olhoft *et al.* 2003; Wang and Ge 2005), and our own selection experiments demonstrated that hygromycin could be used as an efficient selection agent in switchgrass (data not shown). Hygromycin inhibits protein synthesis by impairing proper mRNA translation at the ribosomal A site (Gonzalez *et al.* 1978). The pANIC vector set was constructed with both *bar* and *hph* plant selectable marker genes. We placed each of these genes under the transcriptional regulation of the rice actin1 (*OsAct*1) promoter (McElroy *et al.* 1990) and CaMV 35S terminator. In the binary vectors, these selection cassettes have been placed near the left border of the T-DNA to increase the insertion rate of other cassettes, since transfer of the T-DNA is initiated at the right border.

Reporter Cassettes

Different reporter systems each carry their own advantages or disadvantages, depending on their specific application. Colorimetric reporter systems such as beta-glucuronidase (GUS) can be viewed with the naked eye, eliminating the need for expensive fluorescent excitation capabilities, although they require the addition of a substrate and frequently result in the destruction of tissue. Fluorescent reporter systems such as GFP or DsRed require fluorescent excitation, but provide higher degrees of resolution, require no substrate or cofactor inputs, and allow visualization throughout the life of the plant without destruction of tissue (Stewart 2006). Both colorimetric and fluorescent reporter genes were utilized in the pANIC vector set. For colorimetric assays, the GUSPlus gene from the pCAMBIA vector series (http://www.cambia.org/daisy/cambia/585.html) was used. For fluorescent capabilities, we chose to use the novel red fluorescent protein pporRFP. The pporRFP gene was first described by Alieva *et al.* (2008) and is a DsRed-type of coral fluorescent protein that expresses well in tobacco, *Arabidopsis*, and switchgrass (Figure 11.2).

The constitutive expression of both the GUSPlus gene and the pporRFP gene is driven by the switchgrass ubiquitin 1 promoter (*PvUbi*1), recently isolated and characterized from cv. Alamo. The *PvUbi*1 promoter exhibits strong constitutive expression in dicots and monocots and is active in a broad range of switchgrass tissue types. This promoter contains a three-amino acid fusion from the *PvUbi*1 coding region directly downstream of the intron region to increase accumulation of the transgene product within plants (Hondred *et al.* 1999; Sivamani and Qu 2006). The *PvUbi*1 promoter sequence was cloned upstream in frame with the GUSPlus or pporRFP genes. The *A. tumefaciens* nopaline synthase (NOS) terminator was used for termination of both reporter cassettes.

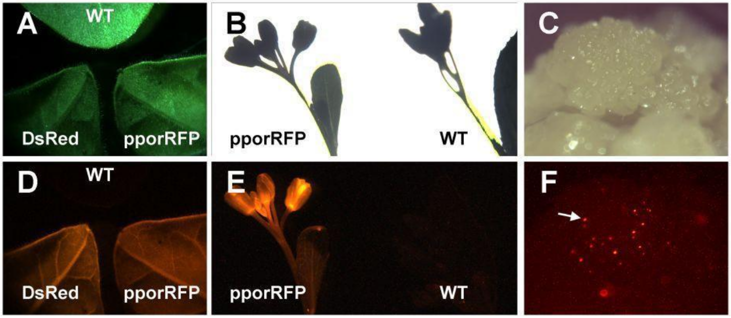

Figure 11.2. Fluorescent imaging of pporRFP expression in plants. (**A**) Brightfield and (**D**) fluorescent images showing pporRFP expression compared with DsRed expression in the leaves of stably transformed tobacco plants compared with wild type (WT). (**B**) Brightfield and (**E**) fluorescent images of wild type and stably transformed *Arabidopsis thaliana* expressing pporRFP. (**C**) Brightfield and (**F**) fluorescent images showing transient pporRFP expression in switchgrass callus following particle bombardment. The arrow indicates a representative of pporRFP fluorescent foci being expressed within the calli. (For a color version of this figure, see Plate 14.)

Distribution

We anticipate that pANIC will be broadly applicable for monocot transformation and the vector set is freely available to noncommercial institutions and is distributed via MTA available here: http://plantsciences.utk.edu/stewart.htm

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