Plant Biotechnology Journal

*Plant Biotechnology Journal* (2012) **10**, pp. 226–236 doi: 10.1111/j.1467-7652.2011.00658.x

# Gateway-compatible vectors for high-throughput gene functional analysis in switchgrass (*Panicum virgatum* L.) and other monocot species

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*Received 31 May 2011;*
*revised 12 June 2011;*
*accepted 10 August 2011.*
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This work was supported by the Bioenergy Science Center. The BioEnergy Science Center is a US Department of Energy Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science.

**Keywords:** switchgrass, DNA vector, transformation, *Agrobacterium*, bombardment, bioenergy, monocot.

## Summary

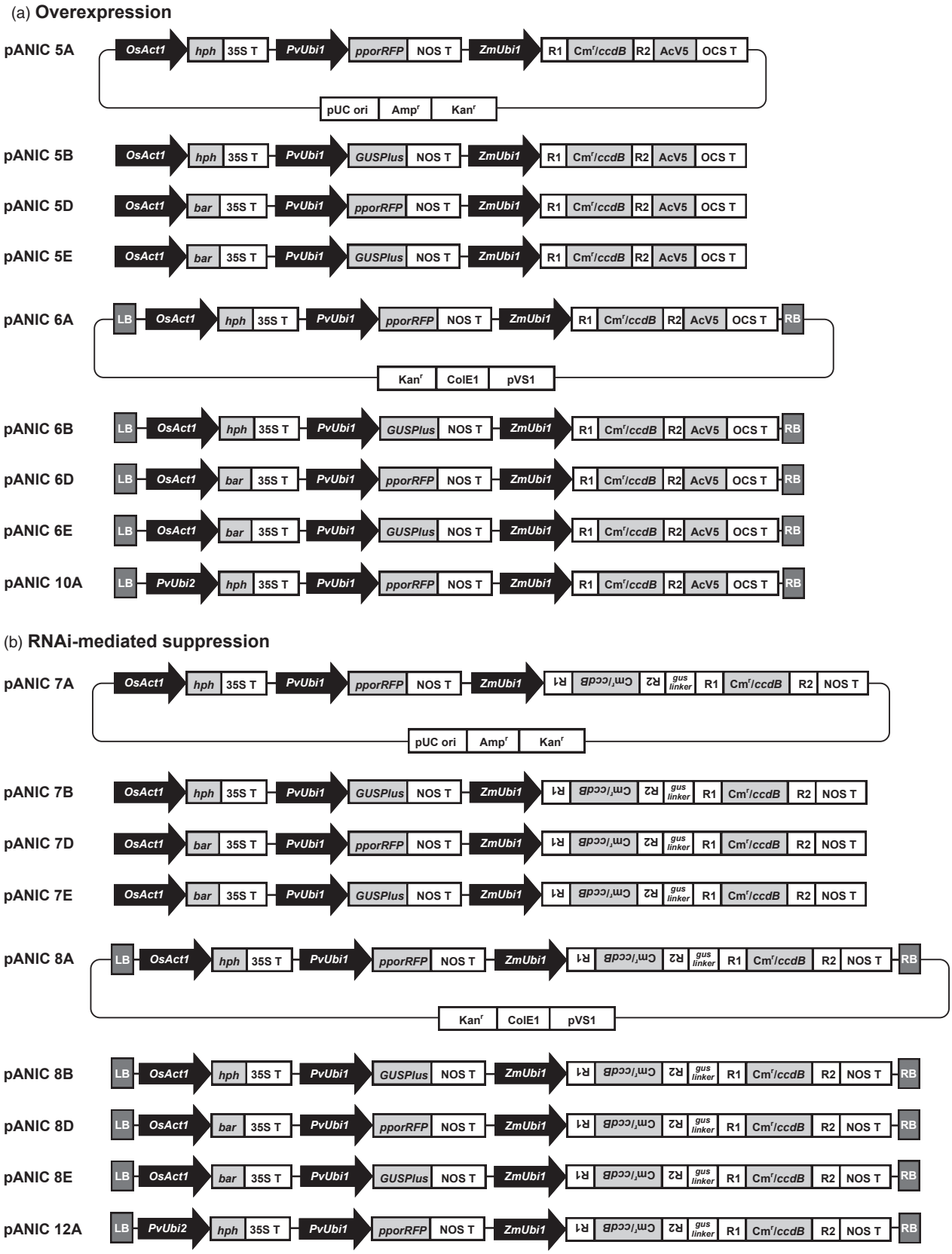
Switchgrass (*Panicum virgatum* L.) is a C4 perennial grass and has been identified as a potential bioenergy crop for cellulosic ethanol because of its rapid growth rate, nutrient use efficiency and widespread distribution throughout North America. The improvement of bioenergy feedstocks is needed to make cellulosic ethanol economically feasible, and genetic engineering of switchgrass is a promising approach towards this goal. A crucial component of creating transgenic switchgrass is having the capability of transforming the explants with DNA sequences of interest using vector constructs. However, there are limited options with the monocot plant vectors currently available. With this in mind, a versatile set of Gateway-compatible destination vectors (termed pANIC) was constructed to be used in monocot plants for transgenic crop improvement. The pANIC vectors can be used for transgene overexpression or RNAi-mediated gene suppression. The pANIC vector set includes vectors that can be utilized for particle bombardment or *Agrobacterium*-mediated transformation. All the vectors contain (i) a Gateway cassette for overexpression or silencing of the target sequence, (ii) a plant selection cassette and (iii) a visual reporter cassette. The pANIC vector set was functionally validated in switchgrass and rice and allows for high-throughput screening of sequences of interest in other monocot species as well.

## Introduction

The development of next-generation sequencing technologies has rapidly increased the amount of nucleotide-based information for many plant species (Varshney *et al.*, 2009), but the generation and study of gain-of-function or loss-of-function mutants is still a requirement for validating gene functionality in higher plants. Various methods exist for elucidating the function of genetic elements in model plant species and applied crop species including virus-induced gene silencing (VIGS) (Ding *et al.*, 2007; Scofield and Nelson, 2009), transient-induced gene silencing (Douchkov *et al.*, 2005; Marzin *et al.*, 2008), transient overexpression assays (Bargmann and Birnbaum, 2009; Saxena *et al.*, 2011), and insertional mutagenesis and activation tagging systems (Pogorelko *et al.*, 2008; Qu *et al.*, 2008; Tadege *et al.*, 2008). However, the stable expression of transgenes and the suppression of native genes *in planta* are the two most common methods for thoroughly understanding the biological role or function of the genes being studied. These methods typically require cloning a sequence of interest into a vector backbone and introducing the DNA into the plant using a variety of transformation methods.

*Agrobacterium*-mediated transformation and particle bombardment are the most widely used methods for transforming plants. *Agrobacterium*-mediated transformation was originally developed for dicotyledonous species, although transformation efficiencies have improved over time for monocotyledonous species using this method (for a review, see Shrawat and Lörz, 2006). Many studies have compared these two methods alongside each other, with *Agrobacterium*-mediated transformation resulting in transgenic plants with lower copy number, lower frequency of transgene silencing, and higher stable expression (Dai *et al.*, 2001; Travella *et al.*, 2005). However, Lowe *et al.* (2009) demonstrated that decreasing the amount of DNA used for particle bombardment can significantly increase the number of single-copy events in maize, although it can negatively affect the transformation frequency. Both methods remain relevant to monocot transformation, and each offers its own advantages and disadvantages.

Many commonly used vector backbones exist for plant transformation methods (for a review, see Komori *et al.*, 2007). Current vector sets have enhanced or replaced older vector backbones (McCormac *et al.*, 1997; Goderis *et al.*, 2002; Thomson *et al.*, 2002), and more advanced plant vectors include reporter gene or epitope tag fusions for tracking gene expression, visualizing protein subcellular localization and other genetic studies (Earley *et al.*, 2006; Chakrabarty *et al.*, 2007; Coutu *et al.*, 2007; Nakagawa *et al.*, 2007), Gateway-compatible sites




for rapid gene cloning (Curtis and Grossniklaus, 2003; Miki and Shimamoto, 2004; Tzfira *et al.*, 2005), and multisite Gateway compatibility for multi-sequence delivery capabilities (Karimi *et al.*, 2007). Gateway technology takes advantage of the bacteriophage lambda site-specific recombination system, facilitating the exchange of DNA fragments from an 'entry' vector to a

**Figure 1** Schematic diagrams of the Gateway-compatible pANIC vectors for overexpression (a) and RNAi-mediated suppression (b). pANIC vectors sharing the same number in their name (5–8) designate that these vectors contain identical backbone sequences. Abbreviations: *OsAct1* (rice actin 1 promoter and intron), *PvUbi1* (switchgrass polyubiquitin 1 promoter and intron), *PvUbi2* (switchgrass polyubiquitin 2 promoter and intron), *ZmUbi1* (maize ubiquitin 1 promoter and intron), *bar* (bialaphos resistance coding region), *hph* (hygromycin B phosphotransferase coding region), *pporRFP* (*Porites porites* red fluorescent protein coding region), *GUSPlus* (GUSPlus™ coding region along with the rice glycine-rich protein signal peptide sequence), $Cm^r$ (chloramphenicol resistance gene), *ccdB* (negative selection marker), 35S T (*35S* terminator sequence), NOS T (*Agrobacterium tumefaciens nos* terminator sequence), OCS T (octopine synthase terminator sequence), AcV5 (epitope tag), LB (left border), RB (right border), R1 and R2 (*attR1* and *attR2* recombination sites), pUC ori and ColE1 (origins of replication in *E. coli*), PVS1 (origin of replication in *A. tumefaciens*), $Amp^r$ and $Kan^r$ (ampicillin and kanamycin bacterial resistance).

'destination' vector in a rapid and efficient manner (Hartley *et al.*, 2000). Available plant transformation vector sets constitute 'destination' vectors and contain the *attR1* and *attR2* recombination sites flanking (i) the chloramphenicol resistance gene ($Cm^r$) and (ii) a negative selectable marker gene (*ccdB*). Overexpression vectors contain the *attR1-*$Cm^r$*-ccdB-attR2* cassette, and RNAi-mediated suppression vectors contain the *attR1-*$Cm^r$*-ccdB-attR2* cassette downstream of an inverted repeat of itself, resulting in a hairpin loop of the target sequence after recombination and transcription. In either case, these Gateway-compatible cassettes are located downstream of an appropriate promoter. The Gateway system allows for rapid recombinational cloning and ease of screening for positive clones.

Many of the available plant vector sets are tailored for functional analysis in dicot species and utilize only a limited number of constitutive promoters to control expression. For instance, the cauliflower mosaic virus (CaMV) *35S* and *2x35S* promoters have been commonly used in vectors to drive expression and/or suppression of genes of interest (Curtis and Grossniklaus, 2003; Earley *et al.*, 2006; Nakagawa *et al.*, 2007). However, the expression of the CaMV *35S* promoter is typically lower in monocots than dicots (Himmelbach *et al.*, 2007; Mazarei *et al.*, 2008; Mann *et al.*, 2011a), and native monocot promoters are more commonly used to transform monocot species (Miki and Shimamoto, 2004; Himmelbach *et al.*, 2007). While the vector sets above are designed for a variety of applications, few of them are suited for high-throughput forward genetics studies in monocots. Himmelbach *et al.* (2007) constructed a Gateway-compatible vector set for monocots with a wide range of flexibility for replacing elements and cassettes, and the *ZmUbi1* promoter driving the target gene cassette showed the highest levels of overexpression; however, the *ZmUbi1* promoter also drives the selection cassette (*hph*), and repeated use of the same promoter for two different transgenes within a single construct can lead to transcriptional gene silencing (De Wilde *et al.*, 2000; Butaye *et al.*, 2005). Novel promoters with strong constitutive expression in monocots and efficient selection cassettes are needed to improve the efficiency of genetic engineering in monocots, as these tools can affect the expression level of the transgene and the transformation efficiency (Prakash *et al.*, 2008). Recent monocot promoters have been isolated and characterized (Sivamani and Qu, 2006; Mann *et al.*, 2011a), but not yet incorporated into and functionally validated in vectors for high-throughput reverse genetic studies in monocots. In this study, we describe the construction and functional validation of a Gateway-compatible vector set that facilitates the optimization of monocot transformation methods and subsequent applications for high-throughput production of stable transgenics in switchgrass and other monocot species.

## Results and discussion

The pANIC vectors (the vectors are named after *Panicum virgatum*, the scientific name for switchgrass) are a versatile set of Gateway-compatible destination vectors for use in overexpression or RNAi-mediated suppression of sequences of interest in higher plant species, particularly monocot species. A preliminary description of the pANIC vector set has been published, but with no performance data (Mann *et al.*, 2011b). All pANIC vectors contain three cassettes (Figure 1). These include (i) a Gateway-compatible cassette for overexpression or suppression of the target gene, (ii) a plant selectable marker cassette for conferring resistance (containing either the *bar* or *hph* gene) to the transformed plant, and (iii) a visual reporter gene cassette (*GUSPlus*™ or *pporRFP*) for optimization of the transformation process, visual tracking of transgenic events and rapid identification of transgenic plants. In addition, all combinations of vector components were also constructed with biolistic and binary T-DNA backbones for flexibility with different plant transformation methods.

### The pANIC vector cassettes

#### *Gateway-compatible cassettes*

To allow for high levels of transgene overexpression and sufficient levels of RNAi-mediated suppression of endogenous genes, the *ZmUbi1* promoter was utilized to drive expression of the Gateway-compatible cassettes. The *ZmUbi1* promoter has been used extensively for expression in a wide variety of monocot plant transformation systems and is a constitutively expressed promoter that regulates gene expression in most tissue types during most stages of plant development (i.e. ubiquitous expression), although some discrepancies have been reported (Cornejo *et al.*, 1993). A previously described Gateway-compatible cassette for overexpression (Earley *et al.*, 2006) was utilized, which contains the AcV5 epitope tag for protein fusions downstream of the Gateway-compatible *attR2* recombination site and has been functionally validated in plants. The Gateway-compatible cassette for RNAi-mediated suppression has been previously described (Miki and Shimamoto, 2004) and is widely used for rice genetic studies. This cassette contains inverted repeats of the Gateway-compatible arrangement (*attR1-*$Cm^R$*-ccdB-attR2*) with an *Escherichia coli*-derived *gusA* linker sequence, creating a hairpin-loop structure that, upon transcription, can be recognized by the RNAi-pathway and employed for targeted gene suppression within the plant. For these RNAi Gateway-compatible systems, regions of the targeted gene (we have used 200–500 bp for switchgrass) can be generated and recombined from entry clones into the destination vector in a high-throughput manner.

*Selectable marker cassettes*

For transformation in monocots, hygromycin B and bialaphos are the two most commonly used selection agents, although a variety of alternatives have been implemented in previous studies (Li *et al.*, 2003; LaFayette *et al.*, 2005; Ochiai-Fukuda *et al.*, 2006). The bialaphos acetyltransferase (*bar*) gene, conferring resistance to bialaphos, was used in initial switchgrass transformation efforts (Richards *et al.*, 2001; Somleva *et al.*, 2002). However, the hygromycin B phosphotransferase (*hph*) gene has been successfully implemented in more recent studies to confer hygromycin B resistance to the transformed tissue (Burris *et al.*, 2009; Xi *et al.*, 2009; Li and Qu, 2011). Therefore, pANIC vectors were designed and constructed with either the *bar* or *hph* plant selectable marker gene to give options to the researcher for the preferred application. Both genes were placed under the transcriptional control of the rice *actin 1* gene (*OsAct1*) promoter (McElroy *et al.*, 1990), although pANIC 10A and 12A contain the switchgrass *ubiquitin 2* (*PvUbi2*) promoter in place of *OsAct1* (described in more detail below) (Figure 1). In the binary T-DNA vectors, the selection cassette was inserted near the left border (LB) to decrease false-positive stable insertions and increase the insertion rate of the gene of interest cassette, because transfer of the T-DNA is initiated at the right border (RB).

*Visual reporter cassettes*

The use of chemical selection agents during transformation allows for positive selection of tissue stably transformed with the DNA of interest. In addition, reporter genes have been shown to significantly enhance the selection process, allowing for rapid identification of stable events, decreasing the number of non-transformed regenerated escapes and reducing the amount of downstream gene-silencing phenomena (Stewart, 2001; Nishizawa *et al.*, 2006; Saika and Toki, 2009). Cotransformation systems can be utilized for introduction of a vector containing a reporter cassette simultaneously with a vector containing the gene of interest, although the actual rate of cotransformation for both constructs can vary. Vectors designed with the reporter gene cassette on the same backbone as the gene of interest cassette could allow earlier identification of stable events for the gene of interest and eliminate issues previously observed with cotransformation.

To accommodate a variety of reporter systems, the pANIC vectors were constructed with *GUSPlus*™, an enhanced derivative of *uidA*, or the *pporRFP* gene isolated from the coral *Porites porites* (Alieva *et al.*, 2008), which produces pporRFP, a DsRed-type fluorescent protein. The *pporRFP* reporter gene was previously validated for switchgrass genetic transformation (Burris *et al.*, 2009), and red fluorescent proteins (RFPs) are advantageous when compared with the green fluorescent protein (GFP) for fluorescence in plants as a result of the lower level of autofluorescence in the red wavelengths (Jach *et al.*, 2001; Dietrich and Maiss, 2002; Nishizawa *et al.*, 2006). The *GUSPlus*™ and *pporRFP* genes are expressed under the control of the switchgrass ubiquitin 1 gene (*PvUbi1*) promoter that has been isolated and characterized for strong constitutive expression in switchgrass and rice (Mann *et al.*, 2011a).

*Biolistic and binary backbones*

Both biolistic and binary T-DNA vector backbones were constructed for the pANIC vector set. All pANIC vectors were originally cloned into the pCR4 backbone for biolistic bombardment (the pANIC 5A-E and 7A-E series), containing the ampicillin (*bla*) and kanamycin (*nptII*) resistance genes for bacterial selection. To facilitate *Agrobacterium*-mediated transformation, the pCR4 backbone was replaced with the pPZP201BK binary T-DNA backbone (Covert *et al.*, 2001) to create an alternate set of binary vectors (the pANIC 6A-E and 8A-E series). The pPZP201BK backbone contains the *nptII* gene for bacterial selection, the ColE1 replication of origin for high copy number replication in *E. coli*, the pVS1 origin for low copy number replication in *Agrobacterium* sp., the pBR322 *bom* site for conjugational transfer, the *rep* and *sta* regions from pVS1 for vector stability in *Agrobacterium* in the absence of selection pressure, and the LB and right border (RB) for *Agrobacterium*-mediated plant transformation (Hajdukiewicz *et al.*, 1994).

## Functional evaluation of the pANIC vectors

To validate the functionality of the Gateway-compatible overexpression cassette, the *gusA* coding region (*GUS*) was recombined into the Gateway-compatible cassette of pCR4B-*ZmUbi1*-GW, the precursor to all overexpression pANIC vectors (see Materials and Methods) under the transcriptional regulation of the *ZmUbi1* promoter and tested for expression via particle bombardment into callus cultures from the switchgrass genotype Alamo 2 along with the pAHC25 positive control vector (Christensen and Quail, 1996) and a negative control (gold particles alone) (Figure 2). The quantitative measurements of the fluorometric assay display similar levels of expression for the *ZmUbi1* promoter in the pANIC vector backbone and the vector of origin (pAHC25). An additional vector was tested for expression levels of GUS under the control of the CaMV *35S* promoter. *GUS* expression levels under the regulation of the CaMV *35S* promoter were significantly lower than those observed under the regulation of the *ZmUbi1* promoter similar to previous observations (Himmelbach *et al.*, 2007; Mazarei *et al.*, 2008).

To test the functionality of the Gateway-compatible RNAi cassette in the pANIC vectors, sequence fragments of approxi-

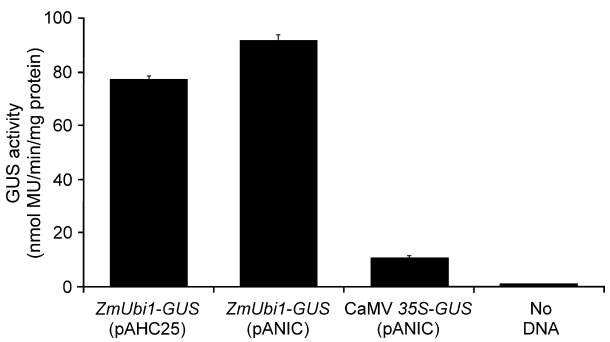



**Figure 2** Quantitative measurements of GUS expression from the pANIC vector Gateway-compatible overexpression cassette using the MUG assay. Because pAHC25 contains the same promoter:gene cassette as the overexpression cassette in the pANIC vector set (*ZmUbi1*:*GUS*), this vector was used as a positive control. Additionally, a pANIC vector overexpression cassette containing the CaMV *35S* promoter was also constructed to test the relative expression levels of the *ZmUbi1* promoter in switchgrass callus cultures. The negative control contained gold particles without the addition of DNA vector. Measurements of *GUS* expression are described in the methods. Data represent the mean value of three independent biological replicates. Bars represent SE.

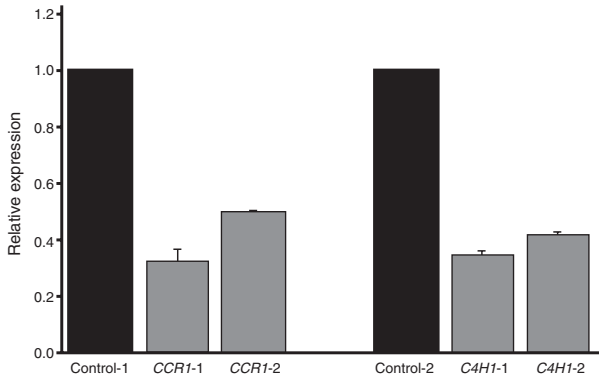



**Figure 3** Expression levels of *PvCCR1* and *PvC4H1* in transgenic switchgrass lines using qRT-PCR. These data demonstrate the RNAi-mediated suppression of *PvCCR1* and *PvC4H1* in two independent lines for each gene using the pANIC 4B vector, a precursor to the pANIC 8B vector (described above). Data represent the mean of three technical replicates. All data were normalized to set the wild-type control at a relative value of 1. Bars represent SE.

mately 400 bp from the switchgrass *cinnamoyl CoA reductase1* (*PvCCR1*) and *cinnamate-4-hydroxylase1* (*PvC4H1*) genes were cloned and recombined into pANIC 4B, a previously cloned precursor to the pANIC 8B vector that contained the *rubi3* promoter in place of the *PvUbi1* promoter for the reporter cassette (Sivamani and Qu, 2006; Mann *et al.*, 2011a). Following switchgrass stable transformation as previously described (Xi *et al.*, 2009), the regenerated transgenic plants were tested for gene expression levels using qRT-PCR (Figure 3). These results demonstrate that the lignin biosynthetic genes *PvCCR1* and *PvC4H1* were down-regulated significantly in the transgenic lines when compared with the wild-type controls, validating that the Gateway-compatible RNAi cassette of the pANIC vector set is effective for suppressing gene expression in switchgrass.

To test the functionality of the *hph* selection cassette under the control of the *OsAct1* promoter, rice (*Oryza sativa* L. japonica) cv. Taipei 309 (TP309) embryogenic callus cultures were transformed with pANIC 7A using particle bombardment. After 3 weeks, stably transformed and hygromycin-resistant rice calli had grown rapidly as compared to the wild-type controls (Figure 4), and at least 33 independent lines of stably transformed rice calli were isolated from the four initial plates that were bombarded. These results demonstrate that the hygromycin selection cassette in the pANIC vector set is functional and that the *OsAct1* promoter is a highly effective promoter for driving hygromycin B resistance in rice transformation. Functional hygromycin selection was also observed while using the pANIC 6A vector for *Agrobacterium*-mediated transformation of switchgrass, and actively growing transgenic calli could be observed on hygromycin selection (hygromycin B 40–60 mg/L) using the *pporRFP* gene as a visual marker (data not shown). Two additional pANIC vectors have been constructed for overexpression (pANIC 10A) and RNAi-mediated suppression (pANIC 12A) with the *PvUbi2* promoter (Mann *et al.*, 2011a) driving the hygromycin B selectable marker gene, as some data suggest that the promoter driving the expression of the selectable marker gene can significantly affect the transformation efficiency and recovery of transformants through the selection process (Prakash *et al.*, 2008; Vogel and Hill, 2008). Both pANIC 10A and pANIC 12A vectors have been functionally validated for gene of interest overexpression/suppression, RFP-expression and hygromycin B selection using similar methods as mentioned above and are available as part of the vector set (data not shown, diagram of vectors displayed in Figure 1).

The reporter cassettes were validated in switchgrass and rice callus using biolistic bombardment assays. The pANIC 7B vector was bombarded into embryogenic rice 'TP309' and switchgrass genotype ST1 callus cultures followed by histochemical staining and visual observation for expression. *GUSPlus*™ expression was observed in rice and switchgrass calli (Figure 5), validating the functionality of the *PvUbi1*:*GUSPlus*™ reporter cassette in the pANIC vectors. To test the functionality of the *PvUbi1*:*pporRFP* reporter cassette, the pANIC 7A vector was bombarded into embryogenic rice TP309 and switchgrass ST1 callus cultures. After 10 days, callus cultures were viewed for detection of fluorescence under a microscope. pporRFP foci were observed in numerous callus pieces for rice and switchgrass (Figure 6). Additionally, real-time tracking of stable transgenic events during different growth stages throughout the tissue culture and

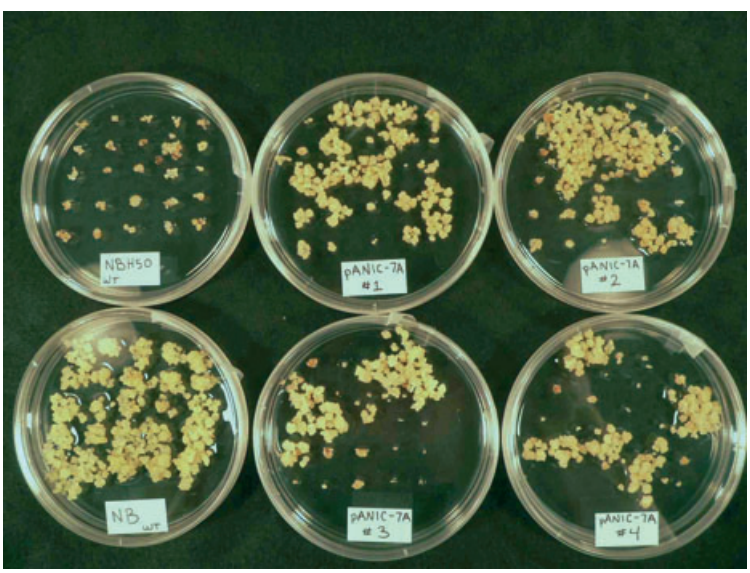



**Figure 4** Selection of transgenic rice calli containing the pANIC 7A vector after 3 weeks on modified NB medium supplemented with hygromycin B at 50 mg/L (MNB-H50). A plate containing non-transformed control tissue on MNB-H50 is shown in the upper left, and a plate containing non-transformed control tissue on NB medium without hygromycin B is shown in the lower left.

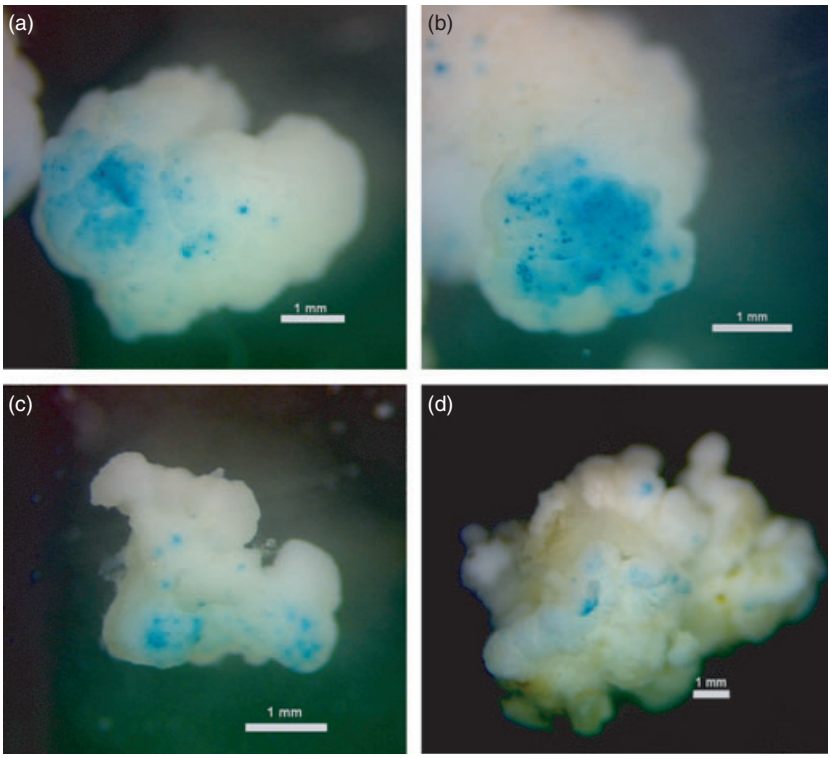



**Figure 5** Histochemical staining for *GUS* expression in rice (a, b) and switchgrass (c, d) calli following particle bombardment with the pANIC 7B vector construct. The measurement bar represents 1 mm.

regeneration process can be observed using *pporRFP* expression (Figure 7). This demonstrates the dual selection capabilities (i.e. antibiotic resistance and visual marker) of the pANIC vector set and the advantage this can offer for optimized stable transformation of monocot species.

## Conclusions

The pANIC series is a versatile set of vectors that has been constructed for stable transformation using *Agrobacterium*-mediated or particle bombardment methods in monocot species and has been validated in rice and switchgrass. This vector set contains Gateway-compatible components for overexpression or RNAi-mediated suppression analyses, as well as multiple combinations of selection marker cassettes and visual reporter cassettes. The Gateway-compatible cassettes allow for convenient insertion of any open reading frame or other target sequence of interest through rapid recombinational cloning. These combined elements provide the researcher with the ability to select the features that are

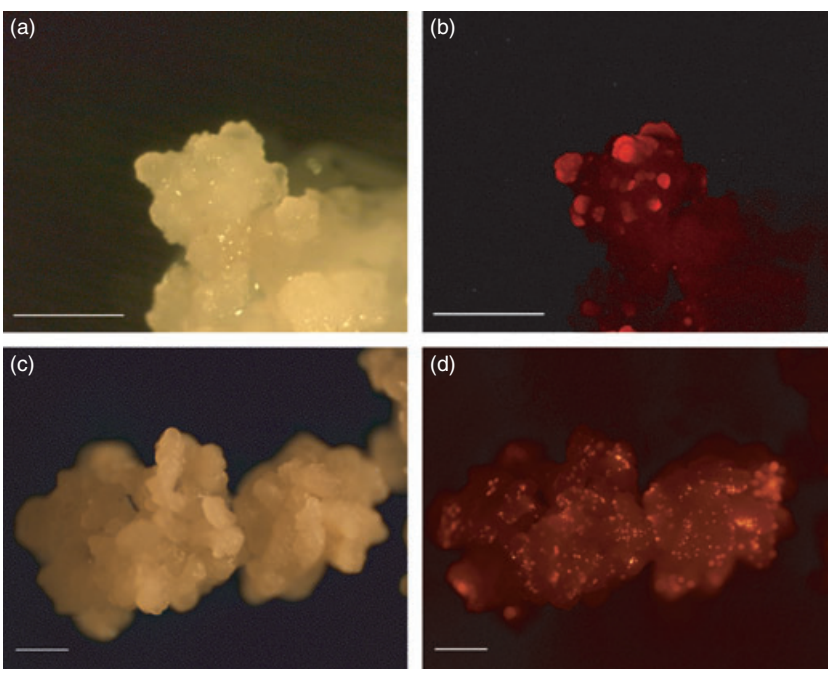



**Figure 6** Fluorescence microscopy showing *pporRFP* expression in rice (a, b) and switchgrass (c, d) calli following particle bombardment with the pANIC 7A vector construct. The measurement bar represents 0.5 mm.

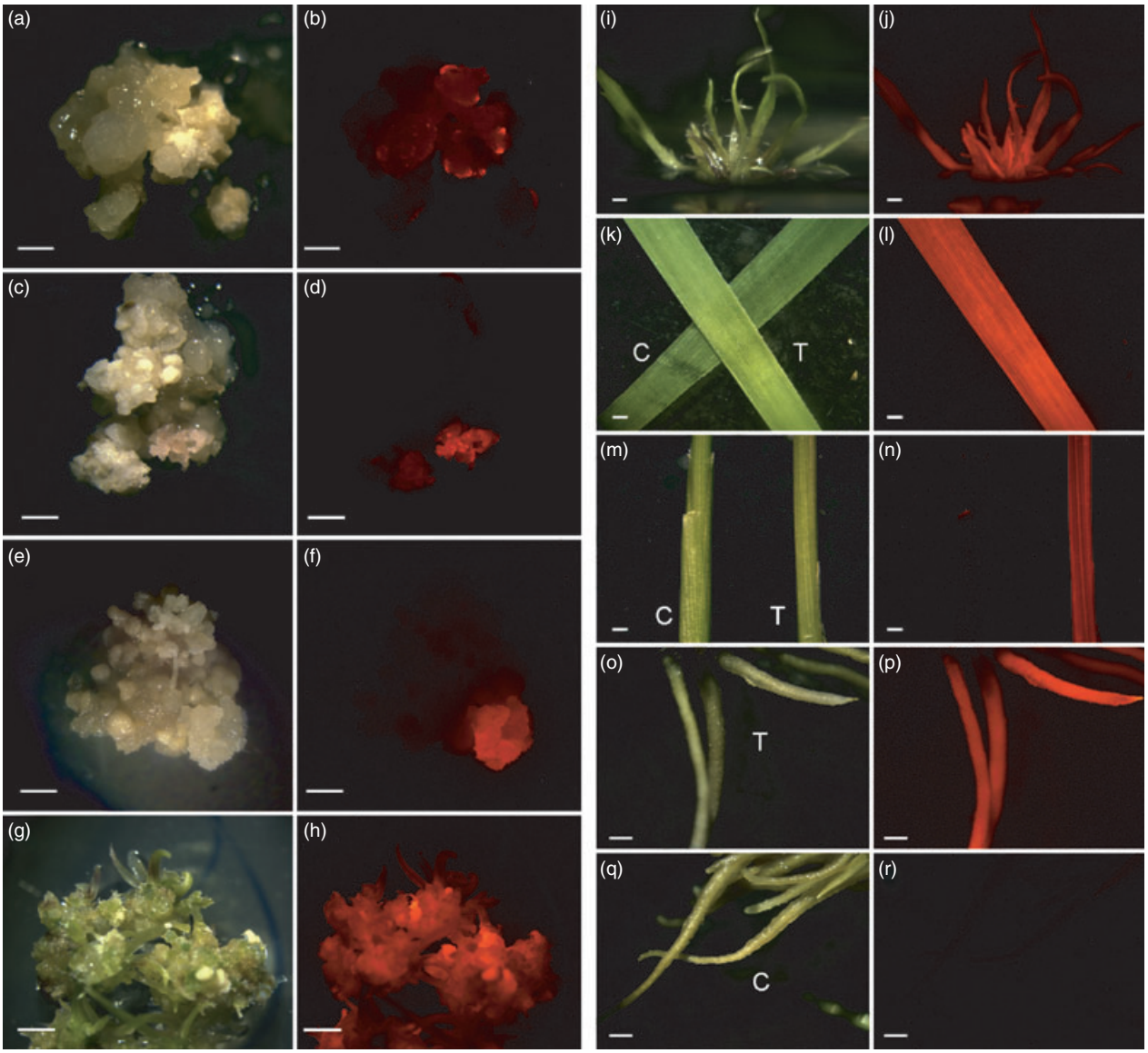



**Figure 7** Real-time tracking of *pporRFP* expression through selection and regeneration of transgenic switchgrass tissues. Expression of *pporRFP* was observed in switchgrass calli at two weeks (a, b) four weeks (c, d) and six weeks (e, f) on hygromycin selection (60 mg/L) following *Agrobacterium*-mediated transformation. Regeneration of fluorescent calli (g, h) resulted in transgenic plantlets expressing *pporRFP* (i, j). Transgenic plants expressed *pporRFP* in leaves (k, l), stems (m, n) and roots (o, p), while non-transgenic plants did not (k, l, m, n, q, r). Measurement bars represent 1 mm. Labels: C, non-transgenic tissue; T, transgenic tissue.

pertinent to their specific transformation system and allow high-throughput production of stable transgenics and functional genomics studies in a particular monocot species. The antibiotic selection (*hph*) in tandem with the fluorescence marker (*pporRFP*) works as a definitive tool for rapid screening and real-time tracking of positive transformation events. This dual selection capability of the pANIC vector set offers an advantage previously unavailable in monocot vector sets. The RFP visualization method also provides a reduction in the amount of time and labour invested in the culturing of tissue during the selection process and no damage or loss of putative transgenic callus tissue or plant organs during the screening process.

The functional validation of the pANIC vectors in rice and switchgrass demonstrates the versatility of these vectors and their components, and these data suggest that the pANIC vector set is a valuable tool for the elucidation of candidate DNA sequence functionality in a broad range of monocot transformation systems. This vector set is freely available for non-commercial research purposes.

## Methods

### Vector construction

The Gateway-compatible overexpression cassette (*attR1*-$Cm^R$-*ccdB-attR2*) coupled with an AcV5 epitope and the *OCS* terminator was PCR amplified from plasmid pEarleyGate 304 (Earley *et al.*, 2006) and cloned into the pCR4B-TOPO backbone (Invitrogen, Carlsbad, CA, USA), creating pCR4B-GW. The *ZmUbi1* promoter was amplified from the pAHC25 plasmid (Christensen and Quail, 1996) using PCR primers supplemented with

*Pst*I and *EcoR*I restriction sites. The PCR product was digested with *Pst*I and *EcoR*I and pCR4B-GW was digested with *Nsi*I and treated with Antarctic Phosphatase (New England Biolabs, Ipswich, MA). The *ZmUbi1* fragment and linearized pCR4B-GW plasmid were ligated together using T4 DNA Ligase (Fisher Scientific, Pittsburgh, PA), creating pCR4B-*ZmUbi1*-GW.

For the Gateway-compatible RNAi hairpin-loop cassette, the plasmid pANDA (Miki and Shimamoto, 2004) was used as a template, although the entire Gateway-compatible hairpin-loop cassette could not be released using restriction endonucleases. Therefore, it was reconstituted as follows: The *NOS* terminator from pCAMBIA 1305.2 (http://www.cambia.org/daisy/cambia/585.html) was PCR amplified and cloned between the *Sac*I and *EcoR*I sites of pMCS03 (a modified pSmart HC-Kan, Lucigen Corp., Middleton, WI) to create pMCS03A. The plasmid pANDA was digested with *Hind*III and *Sac*I, and the *GUS* linker and sense-strand Gateway cassette (*attR1*-Cm$^R$-*ccdB*-*attR2*) region were ligated between the same sites in pMCS03A to create pMCS03B. The *Zmubi1* promoter and antisense-strand Gateway cassette (*attR1*-Cm$^R$-*ccdB*-*attR2*) region from pANDA was released with *Hind*III and cloned into the *Hind*III site of pMCS03B to create pMCS03C. The resulting Gateway-compatible RNAi hairpin-loop cassette was subsequently released from pMCS03C by digestion with *Pme*I and *Asc*I and cloned in place of the Gateway-compatible overexpression cassette in pCR4B-ZmUbi1-GW, creating pCR4B-ZmUbi1-GW-RNAi.

For resistance to hygromycin B or bialaphos, the *hph* and *bar* genes were PCR amplified from pMDC99 and pMDC123, respectively (Curtis and Grossniklaus, 2003), and cloned into the pCR4B-TOPO backbone, creating pCR4B-*bar* and pCR4B-*hph*. The *OsAct1* promoter was excised from pCOR113 (McElroy *et al.*, 1991) using *Acc65*I, filled in with DNA Polymerase I Large (Klenow) Fragment (Promega Corporation, Madison, WI), digested with *Hind*III and gel purified with the QIAquick Gel Extraction kit (Qiagen, Valencia, CA). pCR4B-*bar* and pCR4B-*hph* were digested with *Pme*I and *Hind*III and gel purified, followed by ligation with the *OsAct1* promoter fragment. The resulting plasmids, pCR4B-OsAct1-*bar* and pCR4B-OsAct1-*hph*, were digested with *Pst*I and gel purified. The plasmids pCR4B-*ZmUbi1*-GW and pCR4B-*ZmUbi1*-GW-RNAi were digested with *Sbf*I, treated with Antarctic Phosphatase [New England Biolabs (NEB)] and ligated with the gel-extracted *OsAct1-hph* and *OsAct1-bar* cassettes, resulting in pCR4B-GW-*hph*, pCR4B-GW-*bar*, pCR4B-GW-RNAi-*hph* and pCR4B-GW-RNAi-*bar*, respectively.

For the reporter cassettes, a recently isolated switchgrass ubiquitin promoter (*PvUbi1*) (Mann *et al.*, 2011a) was PCR amplified and cloned directly upstream of the *pporRFP* coding region (Alieva *et al.*, 2008) or the *GUSPlus*™ coding region with the rice glycine-rich protein signal peptide sequence (GRP) from pCAMBIA 1305.2 (http://www.cambia.org/daisy/cambia/585.html). These reporter cassettes (*PvUbi1-pporRFP* and *PvUbi1-GUSPlus*) were digested with *Sca*I and *EcoR*I followed by blunt ending using T4 DNA polymerase (Fisher Scientific). The pCR4B-GW-*hph*, pCR4B-GW-*bar*, pCR4B-GW-RNAi-*hph* and pCR4B-GW-RNAi-*bar* backbones were digested with *Pme*I and treated with Antarctic Phosphatase (NEB). These fragments were blunt-end ligated to each other, creating pANIC 5A-E (overexpression) and pANIC 7A-E (RNAi-mediated suppression) for particle bombardment. For construction of pANIC 6A-E and pANIC 8A-E, the pCR4B-TOPO backbone was removed from pANIC 5A-E and pANIC 7A-E by digestion with *Sbf*I and *Asc*I. The binary backbone T-DNA plasmid pPZP201BK (Covert *et al.*, 2001) was digested with *Pst*I and *Asc*I and ligated to the pANIC 5A-E and pANIC 7A-E inserts, resulting in pANIC 6A-E and pANIC 8A-E. Following cloning, all resulting plasmids were transformed into One Shot® *ccdB* Survival™ 2 T1 Phage-Resistant (T1R) chemically competent *E. coli* cells (Invitrogen) for propagation and plasmid maintenance. All amplified regions and plasmids were sequence verified at the University of Tennessee Molecular Biology Resource Facility.

### Clonase reactions into destination vectors

Sequences of interest were cloned into pCR8/GW/TOPO per the manufacturer's instructions (Invitrogen) and recombined into destination vectors using Gateway® LR Clonase® II enzyme mix (Invitrogen). The resulting expression vectors produced from the recombination reactions were transformed into *E. coli* strain DH5α using standard heat shock method.

### Plant material and growth conditions

Switchgrass cv. Alamo genotypes Alamo 2 and ST1 were maintained and cultured as previously described (Alexandrova *et al.*, 1996; Mann *et al.*, 2011a). ST1 was provided by Zeng-Yu Wang from the Noble Foundation (Xi *et al.*, 2009). Seeds of rice cv. Taipei 309 were provided by the USDA National Plant Germplasm System and maintained and cultured as previously described (Mann *et al.*, 2011a).

### DNA particle bombardment and stable transformation of switchgrass and rice callus

Transient expression assays were performed using biolistic transformation of rice and switchgrass embryogenic callus cultures as previously described (Mann *et al.*, 2011a). For stable transformation of rice, DNA particle bombardment was performed using three-month-old rice callus cultures and 150 ng of plasmid DNA per bombardment. Rice callus cultures were incubated for 6 h pre- and 18 h post-bombardment on 0.6 MNB osmotic medium (Chen *et al.*, 1998). Each vector was used to complete three replicate bombardments, and each replicate consisted of 50 bombarded calli. Rice callus cultures were selected on MNBH50 containing hygromycin B (Calbiochem®, Merck KGaA, Darmstadt, Germany) as described (Chen *et al.*, 1998) to ensure independent events were recovered. For stable transformation of switchgrass, *Agrobacterium*-mediated transformation was performed using previously published protocols (Burris *et al.*, 2009; Xi *et al.*, 2009).

### Isolation of *PvCCR1* and *PvC4H1* fragments for RNAi-mediated suppression

Sequence fragments of approximately 400 bp were selected for *PvCCR1* (from Genbank accession number GQ450304.1) (Escamilla- Treviño *et al.*, 2010) and *PvC4H1* (Hui Shen and Richard Dixon, pers. commun). The primer pairs used for cloning the RNAi fragments are CCR1RNAi.F (5′-AACGGG CAGACCGTGTGC-3′), CCR1RNAi.R (5′-TGCTCCGCCACCGCC TTG-3′), C4H1RNAi.F (5′-CACCCAACGATCCAGTCC-3′) and C4H1RNAi.R (5′-GGTGTCGACCTTGTCCTG-3′). Sequence fragments were cloned into pCR8/GW/TOPO (Invitrogen) and sequence confirmed.

### Quantitative reverse transcriptase-PCR

RNA isolation and reverse transcription were performed as previously described (Shen *et al.*, 2011a). Briefly, 3 μg of

switchgrass total RNA was isolated using the RNeasy Mini kit (Qiagen), treated with DNase (Applied Biosystems, Ambion, Austin, TX) and used for reverse transcription with a reverse transcript III kit (Invitrogen) according to the manufacturer's protocol. The cDNA samples were diluted 20-fold, and 2 μL of the diluted cDNA samples was used as templates for qRT-PCR assays. qRT-PCR and data analysis were as previously described (Karlen *et al.*, 2007). The qRT-PCR primers are CCR1qRT.F (5′-GCGTCGTGGCTCGTCAA-3′), CCR1qRT.R (5′-TCGGGTCATC-TGGGTTCCT-3′), C4H1qRT.F (5′-GGGCAGTTCAGCAACCAGAT-3′) and C4H1qRT.R (5′-CGCGTTTCCGGGACTCTAG-3′).

### Quantification of GUS activity

Fluorometric assays to quantify GUS activity were performed with 4-methylumbelliferyl-β-D-glucuronide (MUG) (Sigma-Aldrich, St. Louis, MO) (Jefferson *et al.*, 1987). Proteins were extracted from calli using GUS extraction buffer consisting of 50 mM sodium phosphate pH 7.0, 10 mM $Na_2EDTA$, 0.1% sodium lauryl sacosyl, 0.1% Triton X-100, and 10 mM β-mercaptoethanol (Sigma). Frozen calli and ice-cold buffer were combined at a 1 : 1 ratio (w/v) and ground in a chilled mortar and pestle. The homogenate was centrifuged for 10 min at 12 000 ***g*** at 4 °C, and the supernatant was removed and centrifuged for an additional 5 min. Clarified extracts were stored at −80 °C prior to analysis. To measure GUS activity, 50 μL of extract was mixed with 450 μL of GUS extraction buffer with 1 mM MUG. An aliquot of 100 μL was removed immediately and added into 1.9 mL stop buffer (0.2 M sodium carbonate) as a control sample. The rest of the mixture was incubated at 37°C. At timed intervals, 100 μL was withdrawn and the enzymatic reaction terminated in stop buffer. GUS activity was determined using microtiter plates and a Synergy HT multi-detection microplate reader (Bio-Tek Instruments Inc., Winooski, VT). A standard curve was prepared with 4-methyllumbelliferone (MU) (Sigma). Protein concentration was determined by the dye-binding method (Bradford, 1972) using a Bradford assay reagent kit (Pierce, Rockford, IL) with bovine serum albumin (Sigma) as a standard. GUS activity is expressed as nmol of MU per min per mg of protein.

### Fluorescent microscopy

Red fluorescent plants and callus tissues were observed using an Olympus SZX12 stereo zoom fluorescent microscope. Filter sets were used for RFP (535/30 nm excitation, 600/50 nm emission) and GFP (475/30 nm excitation, 535/40 nm emission) (Chroma Technology Corporation, Rockingham, VT). Digital imaging was performed using an Olympus QColor 3 CCD camera integrated with QCapture 2.56 imaging software (QImaging Corp., Surrey, BC, Canada). While exposure times varied depending on the intensity of the fluorescent expression in each plant tissue, all fluorescent and bright field images were obtained using identical exposure times for the transgenic and control samples.

All the pANIC vectors described above have been donated to the Arabidopsis Biological Resource Center (ABRC) and are freely available for non-commercial research purposes through ordering online at http://abrc.osu.edu.

## Acknowledgements

The authors gratefully acknowledge Dr Zeng-Yu Wang (Noble Foundation) for providing the switchgrass ST1 plants for tissue culture and transformation. We further thank Dr Mark Curtis for providing plasmids pMDC99 and pMDC123, Dr Keith Earley for providing plasmid pEarleyGate304, Dr Ko Shimamoto for providing plasmid pANDA, Dr Janice Zale and Jason Burris for providing plasmids pAHC25 and pCOR113, and Dr Mikhail Matz for providing the *pporRFP* gene.

## Authors' contributions

DGJM participated in the design, construction and validation of the vectors and drafted the manuscript. PRL participated in the design and construction of the vectors and contributed to manuscript revision. LLA participated in the design of the vectors. ZRK, MM, HB, MCH, CRP and HS participated in experimental design and validation of the vectors. RAD, WAP and CNS were overall study directors and supervisors, participated in the study design, helped to revise the manuscript and obtained the funding. All authors read and approved the final version.

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