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Utilization of a Tobacco Rattle Virus Vector to Clone a *Nicotiana benthamiana* cDNA Library for VIGS

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Virus-induced gene silencing (VIGS) is an efficient and rapid method to identify plant gene functions. One of the most widely used VIGS vectors is *Tobacco rattle virus* (TRV) which has been used successfully for RNA interference (RNAi) in *N. benthamiana* and tomato. We previously modified a TRV VIGS vector to contain the Gateway system for high throughput cloning (Ko *et al.*, J. Fac. Agr., Kyushu Univ., 60(1), 139–149 (2015)), and utilized this system to express a library of *N. benthamiana* cDNA. Random c.300 bp *N. benthamiana* cDNA fragments were generated by ultrasonication and inserted into the TRV VIGS vector by Gateway cloning. *N. benthamiana* were agroinfiltrated with randomly selected TRV cDNA constructs in *Agrobacterium tumefaciens* GV 2260. Distinct visible phenotypes were identified in three sets of the inoculated *N. benthamiana* plants. The three distinguished phenotypes showed leaf malformation and necrosis. The three expressed gene inserts were homologous to EST fragments identified as CK290013.1, CK296346.1, and AM8112161.1, and presumably these genes are related to TRV pathogenesis in *N. benthamiana*. Identification of the selected genes by VIGS will aid further analysis to determine the relationship between VIGS phenotype and gene function.

Key words: *Tobacco rattle virus*, *N. benthamiana* cDNA library, Virus induced gene silencing, Gateway cloning system, Reverse genetics

INTRODUCTION

Studies of plant genome sequences have been completed through advanced sequencing technologies since the genome sequence of *Arabidopsis thaliana* was published (Arabidopsis Genome Initiative 2000). Since then, the complete genome sequences of many crop plant species including rice (Yu *et al.*, 2002), maize (Schnable *et al.*, 2009), soybean (Schmutz *et al.*, 2010), chinese cabbage (*Brassica rapa* Genome Sequencing Project Consortium, 2011), and potato (Potato Genome Sequencing Consortium, 2011) etc., have also been made available.

Although many plant genome sequences are now available, it is often not possible to determine gene function directly from sequence information. Methods to define gene function include overexpression of the gene in transgenic plants, and virus included gene silencing (VIGS) (Burch-Smith *et al.*, 2004). Compared to overexpression in transgenic plants, using VIGS is much more convenient and faster to study gene function. However, VIGS has been limited to testing parts of individual genes or selections from a library. Our previous studies using *Alternanthera mosaic virus* (Ko *et al.*, 2015) showed that insertion of a whitefly cDNA library to an AltMV VIGS

vector was valuable to screen RNAi for control of Whitefly. At the same time we used a natural isolate of Soybean yellow common mosaic virus to clone a *Glycine max* cDNA library for future selection of useful soybean genes (Seo *et al.*, 2015). Through this previous research we have established that a viral vector could be utilized directly as the carrier vector to clone plant and insect cDNA libraries to express RNAi, and we have extended these methods to *Nicotiana benthamiana*.

N. benthamiana is extensively used as a model plant in plant biology, and a draft genome sequence is available (Bombarely A *et al.*, 2012). *N. benthamiana* (family *Solanaceae*) is native to Australia, and significant expressed sequence tag (EST) data is available in addition to the draft genome (Bombarely *et al.*, 2012). Because this species is an efficient host to a broad range of plant viruses, it has been widely used by plant virologists.

Previously, both *N. tabacum* and *N. benthamiana* have been used for protein expression in transgenic plants, or for transient expression using plant viral vectors. At the same time *N. benthamiana* is a good tool for examining viral cell to cell movement and to support plant cell biology based on fundamental science approaches (Chapman *et al.*, 1992; Cruz *et al.*, 1996; Escobar *et al.*, 2003; Lucas, 2006). VIGS can also be used in *N. benthamiana* as a powerful reverse genetic tool to regulate gene expression to identify or confirm gene function (Dong *et al.*, 2007; Fu *et al.*, 2005; Liu *et al.*, 2002; Ratcliff *et al.*, 2001). Especially, VIGS utilizing the RNA interference (RNAi) mechanisms has been developed with several viral vectors for agro-infiltration, including with *N. benthamiana* which is susceptible to

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multiple plant viruses.

VIGS is an efficient and rapid method to identify plant gene functions through knock down gene expression. One of the most widely used VIGS vectors for application in *N. benthamiana* is Tobacco rattle virus (TRV). Since the development of TRV as a VIGS vector, TRV has been successfully used for RNAi in *N. benthamiana* and tomato (Liu *et al.*, 2002a, 2002b). Experimenting on tomato with VIGS, Liu *et al.* had introduced the Gateway system to TRV RNA2 as a VIGS vector for fast and easy cloning (Liu *et al.*, 2002b). The modified TRV VIGS vector was used successfully to test tomato EST function. We have previously constructed our own TRV VIGS vector containing the Gateway system for high throughput use in gene function studies, and to better understand TRV interactions with *N. benthamiana* (Macfarlane, 2010).

Because insertion of random *N. benthamiana* cDNA serves to induce RNAi and thus regulate the homologous endogenous target gene, a phenotype different than a wild type TRV infection of *N. benthamiana* is associated with the function of the target of the TRV cDNA insert. In addition, because the TRV vector itself produces very mild symptoms, increased symptom severity induced by the TRV vector expressing RNAi against a random cDNA insert indicates that the target gene may normally confer partial resistance to TRV infection. Therefore, through expression of an *N. benthamiana* cDNA library from the TRV VIGS vector we were able to identify genes related to TRV pathogenesis in *N. benthamiana*.

In related research we have developed *Soybean yellow common mosaic virus* (SYCMV) as a high throughput soybean gene identification system (Seo *et al.*, 2015), and *Alternanthera mosaic virus* (AltMV) was also developed for efficient cloning and expression of whitefly cDNA RNAi to screen for gene function (Ko *et al.*, 2015). Each of these VIGS vectors was modified with

the Gateway cloning system for easy, efficient construction of large random cDNA libraries.

MATERIALS AND METHODS

INSERTION OF A GATEWAY CLONING ADAPTER IN THE pTRV MCS

To generate a Gateway cloning system in the pTRV multiple cloning site (MCS), a Gateway cloning adapter was amplified from pSITE-2CA vector using primers (Table 1) which added recognition sites for restriction enzymes *SacI* and *XhoI*, at the 5' and 3' ends, respectively. The PCR product and pTRV vector were each digested with *SacI* and *XhoI* and then ligated to generate the pTRV att vector modified with the Gateway cloning system as previously described (Ko *et al.*, 2015).

TOTAL RNA EXTRACTION AND mRNA ISOLATION FROM NICOTIANA BENTHAMIANA

Total RNA was extracted with Trizol reagent (MRC) from leaves of four-week-old *N. benthamiana* and mRNA was isolated with FastTrack[®] MAG mRNA isolation Kit (invitrogen) as described in the manufacturer's recommended protocol, with mRNA eluted in RNase-free water.

CONSTRUCTION OF AN RNAi cDNA LIBRARY USING THE GATEWAY SYSTEM

To construct an *N. benthamiana* cDNA library, we followed the methods of our previous studies (Ko *et al.*, 2015, Seo *et al.*, 2015). Isolated mRNA was used for first strand cDNA synthesis using 3' primer Biotin-attB2-(N)₂₅ (Table 1), and the RNA digested with RNase I. The first strand cDNA was sonicated for 1 second with a Vcx 750 Watt ultrasonic probe to produce cDNA fragments from 250 bp to 500 bp in size. The double stranded 5' attB1-adapter oligomers (Table 1) were ligated to the ends of

Table 1. Oligomers used in this study

Oligomer	Sequence	Feature
Oligomers used in synthesis of cDNA fragments with att site		
5' attB1-adapter	5'-TCGTCGGGGACAACCTTTGTACAAAAAGTTGG-3'	5' attB1-adapter
Biotine-attB2-(N) ₂₅	5'-Biotin-GGGGACAACCTTTGTACAAGAAAGTTGGG(N) ₂₅ -3'	Used for first strand synthesis
Oligomers used in amplifying cDNA		
5' attB1-oligo	5'-TCGTCGGGGACAACCTTTGTACAAAAAGTTGG-3'	5'-Oligo
Biotine-attB2-(N) ₆	5'-Biotin-GGGGACAACCTTTGTACAAGAAAGTTGGG(N) ₆ -3'	3'-Oligo
Oligomers used in sequencing of cDNA library entry clones		
pDONR207_F	5'-TCGCGTTAACGCTAGCATGGATCTC-3'	5'-Oligo
pDONR207_R	5'-GTAACATCAGAGATTTTGAGACAC-3'	3'-Oligo
Oligomers used in PCR and sequencing of cDNA library in Agrobacteria		
pTRV CP 3' end	5'-GAT GCT AAC ATC GCG ACT C-3'	5'-Oligo
pTRV att 3' end	5'-CAT TTG GAT TAC GCT CCA TTT C-3'	3'-Oligo
Oligomers used in Gateway cloning adapter including restriction enzyme site.		
<i>SacI</i> att site F	5'-GAG GAG CTC ACA AGT TTG TAC AAA AAA GCT GAA C-3'	5'-Oligo, <i>SacI</i>
<i>XhoI</i> att site R	5'-GAG CTC GAG ACC ACT TTG TAC AAG AAA GCT G-3'	3'-Oligo, <i>XhoI</i>

the cDNA fragments using T4 DNA ligase, followed by Gateway system cloning. First the cDNA fragments with attached 5' *attB1*-adapter arms were used for synthesis of second strands using Taq DNA polymerase. The double-stranded DNA fragments with *attB1* at 3' ends and *attB2* at 5' ends were then amplified by PCR using a mixture of primers 5' *attB1* and Biotine-*attB2*-(N)₂₅ (Table 1), in order to generate a large quantity of DNA fragments to construct an RNAi cDNA library.

BP RECOMBINATION

The *attB*-flanked amplified cDNA fragments and pDONR 207 vector were used for BP recombination with the BP clonase of the Gateway system. The product of BP reactions was transformed to One shot[®] Top10 electrocompetent cells *E. coli* (Invitrogen). Transformation was performed by electroporator using settings of 1.8kV, 200 Ω , 25 μ F in a final volume of 50 μ l, and then brought to 1 ml with S.O.C. media and incubated in shaking incubator at 37°C. After an 1 hour incubation, 100 μ l of the cDNA library was spread on LB plates containing 1 μ g/ml gentamycin. To determine the cDNA library titer, plates were incubated overnight at 37°C and colonies counted. Twenty four colonies of *E. coli* were randomly selected from the plate and plasmid isolated to determine redundancy and insert size by PCR amplification and fragment analysis on agarose gel. The isolated plasmids which were used for PCR analysis were also sequenced to confirm diversity of the inserted library fragments. The remaining colonies on the plates were harvested to isolate BP library DNA. Then 300 μ l aliquots of the remainder of the cDNA library were mixed with an equal volume of sterile 40% glycerol to create stocks which were stored at -80°C.

LR RECOMBINATION

To generate the TRV *att* cDNA library, the isolated BP library DNA (3 μ g) and modified pTRV *att* vector were mixed and used for LR recombination (Ko *et al.*, 2015). LR reaction products were transformed to One shot[®] Top10 electrocompetent cells *E. coli* (Invitrogen) as above, and plated on LB plates containing 1 μ g/ml kanamycin. All of the colonies on the plates were harvested and the resulting purified TRV *att* cDNA library was transformed into *Agrobacterium tumefaciens* GV2260 and spread on agar plates containing rifampicin and kanamycin. When *Agrobacterium* colonies developed, each of the colonies was analyzed by PCR and sequenced using *Agrobacterium* PCR primers (Table 1) to determine the efficiency of cloning and confirm a low redundancy of the individual constructs (Table 2).

AGRO-INFILTRATION OF *NICOTIANA BENTHAMIANA* WITH THE TRV *att* VECTOR CONTAINING RANDOM cDNA INSERTS

Agrobacterium cultures containing individual TRV *att* cDNA library clones were each mixed with an equal volume of a culture of *Agrobacterium* expressing the TRV RNA 1 construct, and agro-infiltrated to 3–4 week-old *N. benthamiana* plants. To prepare the cultures,

Agrobacterium was incubated at 30°C for 2 days and resuspended in infiltration buffer (10mM MES, (pH 5.6), 10 mM MgCl₂ and 150 mM acetosyringone) at OD600 = 0.6. Equal volumes of the TRV RNA 1 and TRV *att* RNA 2 cultures were mixed, and 5 ml *Agrobacterium* suspension was infiltrated on primary leaves of plants in a single pot using a syringe without a needle.

RESULTS AND DISCUSSION

N. BENTHAMIANA RANDOM cDNA LIBRARY WAS GENERATED USING cDNA SONICATION, PCR AMPLIFICATION AND BP GATEWAY RECOMBINATION

An *N. benthamiana* random cDNA library was generated by cDNA sonication to get small fragments in the range of 250–500 nt, and these small cDNA fragments then amplified by PCR to add Gateway adapters. The cDNA fragments which were prepared for Gateway cloning were inserted into Gateway donor vector pDONR207 using the BP reaction enzyme. BP reaction product entry clones were transformed into *E. coli* and 24 random colonies selected to analyze the inserted cDNA size and sequence. The inserted cDNA size distribution was 200–800 nt (data not shown); the sequences of the selected colonies were analyzed for matches to GenBank gene sequences and EST data. The twenty-four selected colonies all showed distinct sequence insertions; the sequences of eight colonies revealed homologies to *N. benthamiana* or *N. tabacum* mRNA sequences (Table 2). The remainder of the sequenced colonies were not matched to any EST information. Through the electrophoresis and sequence analysis, inserted cDNA fragments were confirmed to have a suitable size and a low redundancy.

TRANSFER OF THE *N. BENTHAMIANA* cDNA TO THE TRV *att* VIGS VECTOR BY GATEWAY LR RECOMBINATION, AND *AGROBACTERIUM* TRANSFORMATION

Entry clones were isolated from harvested BP cDNA library colonies and the inserts transferred by Gateway LR reaction into the TRV *att* vector. The LR reaction products were transformed into *E. coli* and then plasmid DNA purified for transformation into *Agrobacterium* GV2260. *Agrobacterium* colonies were selected and kept individually to study *N. benthamiana* gene function by the Agro-infiltration method. Hundreds of *Agrobacterium* colonies were amplified by PCR to sequence the inserted *N. benthamiana* cDNA. To date the sequences of twenty-nine clones have been analyzed, as shown in Table 3.

REPRESENTATIVE CLONES OF THE *N. BENTHAMIANA* cDNA LIBRARY WERE AGROINFILTRATION TO PLANTS TO CONFIRM THE UTILITY OF THE TRV *att* VIGS VECTOR FOR GENE FUNCTION IDENTIFICATION

Twenty-nine single colonies of the TRV *att* vector containing *N. benthamiana* cDNA fragments were individually agro-infiltrated to first true leaves of 3–4 week

Table 2. Identification of 24 BP reaction clones of *N. benthamiana* cDNA library analysed for redundancy and insert sequence against EST data

No.	Acc No.	EST data	Range (Identities)
BP 1		Unknown	
BP 2		Unknown	
BP 3	GO603828.1	NBERO1CH_T3_042_A06_09JUL2006_048 NBERO1CH <i>Nicotiana benthamiana</i> cDNA, mRNA sequence	339–589 (89%)
BP 4		Unknown	
BP 5	CK295035.1	EST757749 <i>Nicotiana benthamiana</i> mixed tissue cDNA library, normalized, full-length <i>Nicotiana benthamiana</i> cDNA clone NBMCR96 5– end, mRNA sequence	128–271 (94%)
BP 6		Unknown	
BP 7		Unknown	
BP 8		Unknown	
BP 9		Unknown	
BP 10	FG638375.1	TT–47_L15 Samsun trichome library <i>Nicotiana tabacum</i> cDNA, mRNA sequence	134–283 (84%)
BP 11	GO603039.1	NBERO1CH_T3_033_A05_31JUL2006_047 NBERO1CH <i>Nicotiana benthamiana</i> cDNA, mRNA sequence	136–312 (89%)
BP 12		Unknown	
BP 13	CN744195.1	SAL_US027xd22r1.ab1 SAL_US N.Bentheme <i>Nicotiana benthamiana</i> cDNA 3–, mRNA sequence	386–565 (99%)
BP 14	FG195117.1	AGN_PNL222df1_c12.trimmed.seq AGN_PNL <i>Nicotiana tabacum</i> cDNA 5–, mRNA sequence	619–809 (82%)
BP 15	CN745018.1	SAL_US007xh21r1.ab1 SAL_US N.Bentheme <i>Nicotiana benthamiana</i> cDNA 3–, mRNA sequence	179–387 (100%)
BP 16		Unknown	
BP 17		Unknown	
BP 18		Unknown	
BP 19	AM783806.1	AM783806 seedling library, SL <i>Nicotiana tabacum</i> cDNA clone nt002154046, mRNA sequence	114–386 (83%)
BP 20		Unknown	
BP 21		Unknown	
BP 22		Unknown	
BP 23		Unknown	
BP 24		Unknown	

old *N. benthamiana* seedlings. Individual constructs were inoculated to 3 plants, and a distinctive visible phenotype was observed at about 2 weeks after inoculation (Figure 1). Constructs Agro 29 (CK290013.1), Agro 30 (CK296346.1) and Agro 60 (AM811216.1) induced leaf malformation and necrosis (Figure 1). We have not identified any published reports relating to CK290013.1 (Agro 29) or CK296346.1 (Agro 30) to support our observation

of leaf malformation and necrosis. However, AM811216.1 was previously examined in *N. tabacum* to identify essential genes differentially expressed in leaves (Wolfgang *et al.*, 2008); this EST was extracted from plants after cold treatment to induce genes under oxidative stress. Reactive oxygen species (ROS) caused by plant pathogen or environmental stress induces cell death (Kiselevsky *et al.*, 2014). Necrosis of leaves which were inoculated with

Table 3. Agrobacterium colony PCR and BLAST search data of inserts of TRV VIGS constructs from the *N. benthamiana* cDNA library

No.	Acc No.	EST Data	Range (Identities)
Agro 11		Unknown	
Agro 15		Unknown	
Agro 27		Unknown	
Agro 29	CK290013.1	EST752735 <i>Nicotiana benthamiana</i> mixed tissue cDNA library, normalized, full-length <i>Nicotiana benthamiana</i> cDNA clone NBMBR88 5' end, mRNA sequence.	578–818 (98%)
Agro 30	CK296346.1	EST759060 <i>Nicotiana benthamiana</i> mixed tissue cDNA library, normalized, full-length <i>Nicotiana benthamiana</i> cDNA clone NBMD115 5' end, mRNA sequence.	444–608 (86%)
Agro 31		Unknown	
Agro 33	ES886892.1	NBT042_2005–09–17_1/NBT042_D04_1 <i>Nicotiana benthamiana trichomes Nicotiana benthamiana</i> cDNA, mRNA sequence.	1–147 (98%)
Agro 39	GO603828.1	NBERO1CH_T3_042_A06_09JUL2006_048 NBERO1CH <i>Nicotiana benthamiana</i> cDNA, mRNA sequence.	111–391 (91%)
Agro 40		Unknown	
Agro 46	GO608093.1	NBERO1CH_T3_092_A05_15JUL2006_047 NBERO1CH <i>Nicotiana benthamiana</i> cDNA, mRNA sequence.	77–218 (90%)
Agro 47		Unknown	
Agro 60	AM811216.1	AM811216 COL, cold overnight library <i>Nicotiana tabacum</i> cDNA clone nt006018042, mRNA sequence.	209–385 (100%)
Agro 66		Unknown	
Agro 70		Unknown	
Agro 73		Unknown	
Agro 75	AM804954.1	AM804954 seedling library, SL <i>Nicotiana tabacum</i> cDNA clone nt002307093, mRNA sequence.	2–202 (100%)
Agro 77		Unknown	
Agro 79		Unknown	
Agro 83		Unknown	
Agro 85	GO606553.1	NBERO1CH_T3_074_C06_12JUL2006_044 NBERO1CH <i>Nicotiana benthamiana</i> cDNA, mRNA sequence.	426–600 (100%)
Agro 87		Unknown	
Agro 93		Unknown	
Agro 94		Unknown	
Agro 95		Unknown	
Agro 96		Unknown	
Agro 97		Unknown	
Agro 98		Unknown	
Agro 99		Unknown	
Agro 100		Unknown	

construct No. Agro 60 was therefore presumed to relate to silencing of an oxidative stress response gene. While this construct sequence was identified by a Blast search on nucleotide data, this gene is known to be encoded by the chloroplast genomic DNA of *N. benthamiana*. No. Agro 29 also was analyzed by Blast search of nucleotide data that identified it as most closely related to the promoter region of the chromomethylase 3 (CMT3) gene of *N. benthamiana*. No. Agro 30 was not matched to any available nucleotide data.

Plant viral vectors may be the most efficient method to obtain initial information about possible gene function of unknown sequences, being far faster than generation of transgenic plants or selection of knockout mutants. A potential disadvantage is that unlike a knockout mutant, VIGS knockdown may be only partial, and may be insufficient to produce a visible phenotype. However, VIGS has a distinct advantage over transgenic plants or knockout mutants, in that expression of genes essential in early development (for which mutants may be lethal, and transgenics or knockouts thus not viable) can be reduced by VIGS in the developed plant.

We have demonstrated through our VIGS research that the genes represented by the constructs Agro 29, 30, and 60 appear to be strongly related to viral pathogenesis or normal plant development. Further investigation of the selected genes screened by VIGS will be necessary to understand the interactions between TRV and *N. benthamiana*, and how these genes affect the host defenses of *N. benthamiana* against TRV. It is, however, clear from this work that the TRV att VIGS vector has great utility to reveal plant gene function in a high

throughput system.

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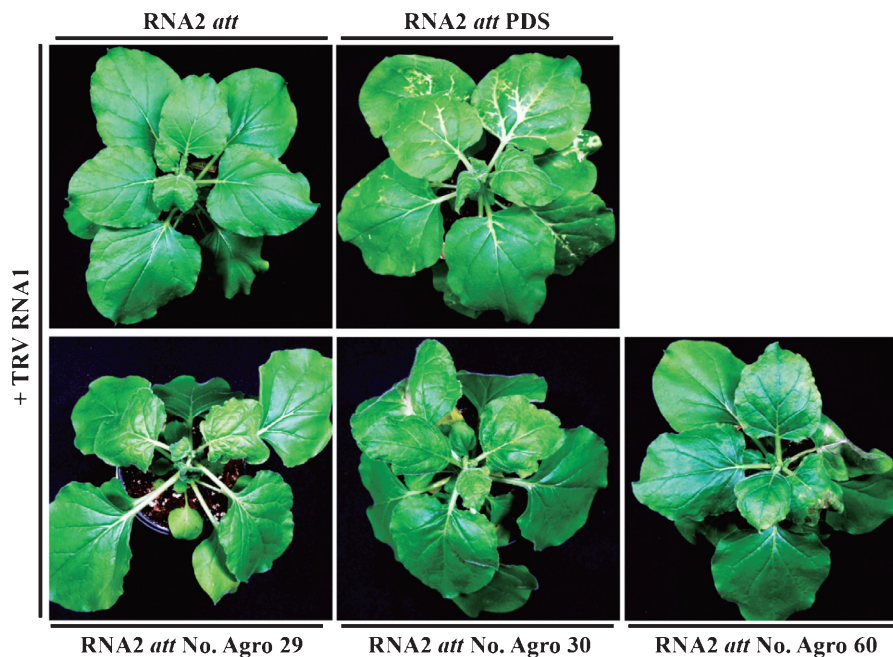


Fig. 1. Phenotype of inoculated *N. benthamiana* producing obvious visible phenotypes caused by TRV induced gene silencing. Plants were infiltrated with TRV att vector, TRV att PDS (phytoene desaturase gene) and TRV att library constructs. *N. benthamiana* infected with TRV att VIGS Nos. Agro 29, Agro 30, Agro 60 caused necrosis, leaf malformation and growth inhibition. Other plants infected with TRV att VIGS construct had typically viral symptom and leaf malformation but less severe symptoms (picture not shown).

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