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https://doi.org/10.5109/1526309

出版情報：九州大学大学院農学研究院紀要. 60 (1), pp.139-149, 2015-02-27. Faculty of Agriculture, Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **60** (1), 139–149 (2015)

Developing an *Alternanthera Mosaic Virus* Vector for Efficient Cloning of Whitefly cDNA RNAi to Screen Gene Function

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(*Received October 31, 2014 and accepted November 14, 2014*)

Plant viral vectors have shown significant promse for studies of gene function, through either up–regulation or down–regulation of gene expression. However, there have remained issues of efficiency of generating constructs, and of subcellular localization of expression; both issues are addressed here. *Alternanthera mosaic virus* (AltMV; genus *Potexvirus*) is distinguished from the type member of the genus, *Potato virus X* by features of viral movement and variation within triple gene block protein 1 (TGB1). AltMV TGB1 variants TGB1L88 and TGB1P88 confer strong and weak silencing suppression, respectively, depending on the presence of L or P at residue 88. Because AltMV replication is associated with chloroplasts, we compared the relative efficiency of RNA interference (RNAi) vectors derived from AltMV and *Tobacco rattle virus* (TRV) to silence a chloroplast–encoded gene. An AltMV RNAi vector expressing a fragment of the chloroplast β ATPase gene reduced β–ATPase expression 1.5 times more than the TRV RNAi vector expressing the same fragment. In addition, we used AltMV (TGB1P88) to create a whitefly (*Bemisia tabaci*) RNAi vector. For this purpose, we first introduced the Gateway cloning cassette into the AltMV multiple cloning site, into which polymerase chain reaction (PCR) products from a whitefly cDNA library could be easily cloned. Second, a mixture of five different PCR fragments of about 250 bp were used to test cloning efficiency of the newly–created AltMV–P–att vector. Third, random 250 bp fragments of Gateway cDNA libraries from *B. tabaci* and *Nicotiana benthamiana* were efficiently cloned into the Gateway–modified AltMV–att vector, demonstrating for the first time a high throughput RNAi system based on AltMV. This strategy could be applied to other RNAi systems.

Key words: *Alternanthera mosaic virus*, Gateway cloning system, *Bemisia tabaci*, RNAi, *Nicotiana benthamiana*

INTRODUCTION

The up–regulation or down–regulation of gene expression is very important for the identification of gene function, a cornerstone of targeted plant breeding and selection. Developing technologies such as RNA interference (RNAi) contribute significantly towards this goal, but use of available plant viral vectors has been limited by the resources necessary to to prepare and utilize the necessary gene constructs, and to some extent by the tissue or subcellular localization of the altered gene expression. Here we demonstrate not only significantly improved efficiency of construct preparation and delivery, but also an improvement in the silencing of a chloroplast–encoded gene.

Since the discovery that plant RNA viruses alter host RNA–based gene silencing pathways, plant RNA viruses have been used as vectors for virus–induced gene silencing (VIGS). Originally VIGS was developed to exploit an RNA–mediated antiviral defense mechanism, and it has been used for plant reverse genetics (Godge *et al* 2008). Both *Tobacco rattle virus* (TRV) and *Barley stripe mosaic virus* (BSMV) have been widely applied for to define host genes function (Burch–Smith *et al*., 2004; Scofield and Nelson, 2009; Cakir *et al*., 2010). Using a VIGS vector, it is possible to knock out a target mRNA of unknown function by expressing a fragment of the mRNA in the vector. Gene silencing results from virus–produced small interfering RNA (siRNA), which produces RNA interference (RNAi) of the target gene sequence, which can yield a different phenotype compared to the wild type (Baulcombe, 2004). Compared to producing transgenic plants, utilizing the recently developed VIGS tools to examine gene function saves considerable time (Scofield and Nelson, 2009). Double stranded RNA generated by plant RNA virus replication induces post transcriptional gene silencing (PTGS), which is the process through which VIGS functions. (Baulcombe, 2004). However, many plant RNA viruses express proteins that allow them to overcome host PTGS in order to replicate their RNA efficiently. For example, helper component–protease (HC–Pro, potyviruses), 2b protein (cucumoviruses), and triple gene block 1 (TGB; potexviruses, hordeiviruses) suppress PTGS in host plants (Brigneti *et al*., 1998; Voinnet *et al*., 2000). Meanwhile, in order to develop appropriate VIGS vectors for each

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host, suitable plant viruses have been selected and modified to be more effective for PTGS by measures such as weakening of the viral silencing suppressor function (e.g. Lim *et al*., 2010a).

Several reports have demonstrated that chloroplast interactions are required for replication of some viruses (Prod'homme *et al*., 2003; Torrance *et al*., 2006). Recent findings show that *Bamboo mosaic virus* (BaMV) host factor chloroplast phosphoglycerate kinase (PGK) interacts with BaMV viral RNA (Lin *et al*., 2007) to aid the transport of viral RNA to the chloroplast to maintain its replication (Cheng *et al*., 2013). The size of the chloroplast genome is about 120,000–170,000 base pairs, and the function of about forty chloroplast proteins are known (Dann and Leighton 2002; Clegg *et al*., 1994). Reinero and Beachy (1986) showed that TMV coat protein (CP) accumulated in tobacco chloroplasts, while Schoelz and Zaitlin (1989) found that TMV genomic RNA but not subgenomic RNA enters chloroplasts, and suggested that CP detected inside chloroplasts is translated by chloroplast ribosomes due to presence of a Shine–Delgarno sequence upstream of the CP gene. Other viruses are also known to interact with chloroplasts, and some of the more than 75 chloroplast–encoded proteins (e.g. Kim *et al*., 2006) have been shown to interact with various viral proteins associated with replication, movement, or symptom expression. VIGS has been utilized as a tool to further examine chloroplast gene function, most commonly with the TRV vector, but a VIGS vector which itself associates with chloroplasts may prove more effective for VIGS of chloroplast genes.

Recently, Lim *et al*. (2010b) developed a new versatile VIGS vector using *Alternanthera mosaic virus* (AltMV), which has a naturally occurring variant in the TGB1 silencing suppressor. AltMV therefore has either an efficient silencing suppression function when TGB1 residue 88 is leucine (TGB1–L), or weak silencing suppression when residue 88 is proline (TGB1–P). The vector with TGB1–L is therefore valuable for high level protein expression, whereas TGB1–P is useful for efficient VIGS (Lim *et al*., 2010a). AltMV replication is associated with chloroplasts, while AltMV TGB3 targets the chloroplasts (Lim *et al*., 2010b), and strongly interacts with chloroplast oxygen evolving protein PsbO (Jang *et al*., 2013). In situ hybridization revealed that AltMV RNA was associated with chloroplasts (Lim *et al* 2010b). In addition, AltMV has a wide host range including tomato, spinach and soybean, and the model plants *Arabidopsis thaliana* and *Nicotiana benthamiana* (Hammond *et al* 2006; Lim *et al*., 2010a). Therefore, AltMV will be useful to study reverse genetics and protein expression in various hosts.

VIGS is an excellent tool for the study of gene function using reverse genetics methods with a VIGS vector; AltMV *Apple latent spherical virus* (ALSV), BSMV, and TRV have been applied to examine gene function. The requirement for VIGS to be used for genetic studies is that the vector should be applicable to various hosts; these four viruses have been qualified for specific requirements. The host ranges of TRV, BSMV, ALSV, and AltMV include some important crop plants such as tomato, wheat, apple, and soybean, as well as the model plant *N. benthamiana*, and these VIGS vectors typically induce mild or undetectable symptoms, making them useful in a number of important host systems.

Because VIGS can be applied to identify the function of an unknown gene, it should be useful for crop improvement. However, there have been limited applications to understand gene function through high–throughput reverse genetics tools. In the case of soybean, 63,670 non–redundant expressed sequence tags (ESTs) of soybean have been reported (Hisano *et al*., 2007), and similar number of ESTs are to be found in other economically important crops. In most crops the use of VIGS for reverse genetic studies to date has been limited to examination of specific genes, rather than the investigation of function of large numbers of genes.

Here, we report a new Gateway–modified AltMV vector to more easily insert foreign genes. Furthermore, we used *Bemisia tabaci* genomic RNAi randomly inserted to AltMV to screen for gene function, and demonstrated the potential to identify unknown gene function using this system.

MATERIALS AND METHODS

CONSTRUCTION OF AN AltMV GATEWAY CLONING VECTOR

To construct a Gateway cloning vector, a Gateway cassette was amplified by polymerase chain reaction (PCR) using primers *att*R1_F_*Bgl*Ⅱ and *att*R2_R_*Nhe*I (Table 1). Then the PCR product was inserted into the pUC MCS vector (Lim *et al*., 2010a) digested with *Bgl*Ⅱ and *Nhe*I (the pUC MCS vector includes a portion of TGB2, and all of TGB3, multiple cloning site (MCS) site, coat protein gene and 3' non–coding region of AltMV). Subsequently, AltMV MCS (vector including AltMV RdRp, TGB1, and a portion of TGB2 coding sequences of AltMV followed by an MCS) and pUC MCS : *att*R were each digested with *Xma*I and *Xba*I. The 3' part of pUC MCS : *att*R was inserted into AltMV MCS, followed by the remaining *Xma*I fragment, creating AltMV–*att*R. After ligation and sequencing to confirm the correct structure, AltMV–*att*R could be used for Gateway cloning.

CONSTRUCTION OF A TRV GATEWAY CLONING VECTOR

To construct a TRV Gateway cloning vector, the Gateway cassette containing 5' *Sac*I and 3' *Xho*I recognitions sequences was amplified essentially as described above using primers *att*R1_F_*Sac*I and *att*R2_R_*Xho*I (Table 1). This cassette was inserted into pYL156:TRV2 between the *Sac*I and *Xho*I sites. As with the AltMV–*att*R Gateway cloning vector, pYL156:TRV–*att*R could also be used of Gateway cloning.

TOTAL RNA EXTRACTION AND mRNA ISOLATION FROM *BEMISIA TABACI* AND CONSTRUCTION OF A *BEMISIA TABACI* CDNA LIBRARY IN GATEWAY SYSTEM FOR RNAi

The whitefly *B. tabaci* was reared on tomato plants

(*Lycopersicon esculentum*) in an insectarium (25 ± 1˚C, 55 ± 5% RH, 16:8 (L:D) photo period). Total RNA was extracted from 5 mg of adult *B. tabaci* using Trizol reagent (MRC) and mRNA was concentrated by using FastTrack® MAG mRNA isolation kit (Invitrogen), following the manufacturer's protocol. Purified mRNA was used to synthesize first strand cDNA using random primer Biotin–*att*B2–$(N)_{25}$ (Table 1) and SuperScript® Full length cDNA Library Construction Kit Ⅱ protocol with some modifications (see below). The primer was designed to include the *att*B2 sequence, with $(N)_{25}$ added in order to allow the primer bind to mRNA at random locations. Then the first strand cDNA was sonicated with a Vcx 750 Wat*t* ultrasonic processor (Sonics & Materials) for 1

Table 1. Primers used in this study.

Clone	5'–Oligo	5'–Oligo sequence	3'–oligo	3'–Oligo sequence
Primers used in cloning vector construction				
AltMV vector	*att*R1_F_*Bgl*II	ACAAGTTTGTACAAAAAAGCTGAAC	*att*R2_R_*Nhe*I	ACCACTTTGTACAAGAAAGCTG
TRV vector	*att*R1_F_*Sac*I	GAGGAGCTCACAAGTTTGTACAAAAAAGCTGAAC	*att*R2_R_*Xho*I	GAGCTCGAGACCACTTTGTACAAGAAAGCTG
Primers used in cDNA library construction				
First_strand synthesis			Biotin–*att*B2–$(N)_{25}$	Biotin–GGGGACAACTTTGTACAAGAAAGTTGGG(N)25
*Att*B1_adapter ligation (double–strand)	*att*B1–adapter–U	TCGTCGGGGACAACTTTGTACAAAAAAGTTGG		
	*att*B1–adapter–L	pCCAACTTTTTTGTACAAAGTTGTCCCC		
*att*B–flanked DNA	*att*B1–adapter–U	TCGTCGGGGACAACTTTGTACAAAAAAGTTGG	Biotin–*att*B2–$(N)_6$	Biotin–GGGGACAACTTTGTACAAGAAAGTTGGG$(N)_6$
	*att*B1–adapter–U	TCGTCGGGGACAACTTTGTACAAAAAAGTTGG	Biotin–*att*B2–$(N)_{25}$	Biotin–GGGGACAACTTTGTACAAGAAAGTTGGG$(N)_{25}$
Primers used in BP recombination assay				
Whitefly cDNA	pDONR 207 F	TCGCGTTAACGCTAGCATGGATCTC	pDONR 207 R	GTAACATCAGAG*Att*TTGAGACAC
Primers used in Gateway Cloning Efficiency Test assay				
N. benthamiana GSTU4	GSTU4 F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTATGTTGATAAAAAGAT	GSTU4 R	GGGGACCACTTTGTACAAGAAAGCTGGGTCAGCAAGAGACTTTGGA
N. benthamiana Ankyrin	Ankyrin F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCAGTATGGTCACTGGGA	Ankyrin R	GGGGACCACTTTGTACAAGAAAGCTGGGTCGCCGTTCAGCGCTGCCA
N. benthamiana bHLH2	bHLH2 F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCG*Att*TCACGACCCACTC	bHLH2 R	GGGGACCACTTTGTACAAGAAAGCTGGGTCCCAAATCGGGCTTGAGC
N. benthamiana XI–K F	XI–K F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGTAA ACCCTTTCCAAAG	XI–K R	GGGGACCACTTTGTACAAGAAAGCTGGGTCAAATGCTTCAAGAACGG
B.tabaci Chickadee	Chickadee F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTTAAGTTTTCAAAATGA	Chickadee R	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTGCTTGCTGAGGTTGA
LR_insert sequence analyze			*att*R2 R	ACCACTTTGTACAAGAAAGCTG
Primers used in QRT–PCR				
QRT–PCR *N. benthamiana* Actin	QRT_Nb_Actin_F	*Att*GTCAGCAACTGGGATG	QRT_Nb_Actin_R	CACG*Att*AGCCTTTGGGTTA
QRT–PCR *N. benthamiana* beta ATPase	QRT_Nb_ATPase_beta_F	TGGCAAGAGGTCAACGAT	QRT_Nb_ATPase_beta_R	CCTGTCCAACTTCTAATGAATCA

sec to produce cDNA of 250 bp to 500 bp, and fragments collected following ethanol precipitation. These small cDNAs were ligated to the double–strand 5' *att*B1 adapter (*att*B1–adapter–U annealed with *att*B1–adapter–L; Table 1) using T4 DNA ligase, for use in subsequent Gateway system cloning. Second strand cDNA was synthesized with LA Taq™ DNA polymerase (Takara). The *att*B–flanked DNA was amplified with a mixture of Biotin–*att*B2–$(N)_{25}$ and Biotin–*att*B2–$(N)_6$ with *att*B1–adapter–U (Table 1). The amplified fragments were prepared using a PCR purification kit, and used for BP recombination (Figure 1).

BP RECOMBINATION

4.8 μg of the purified *att*B–flanked cDNA PCR product was inserted into 4.5 μg of vector pDONR™ 207 (Invitrogen) by Gateway BP recombination. The product of BP recombination was eluted with 10 μl of distilled water. The products of recombination were used to transform 50 μl of One shot® Top10 electrocompetent *Escherichia coli* cells (Invitrogen) by electroporation with 1.5 μl of cDNA library sample. The entire contents of the tube was transferred to a pre–chilled 0.1 cm cuvette and electroporated immediately at 1.8 kV, 200 Ω, and 25 μF. One ml of S.O.C medium was added to the cuvette containing electroporated cells, which were shaken for at least 1 hour at 37°C at 225–250 rpm to allow expression of the gentamycin resistance marker. Next, 100 μl of the cDNA library was serially diluted with 900 μl of LB broth to a dilution of 10^{-4} and spread on LB plates containing 1 ug/ml gentamycin, and the plates were incubated overnight at 37°C .The cDNA library titer was determined by counting colonies. We analyzed plasmids from 20 randomly selected colonies on agarose gel for insert size. We confirmed cDNA insertions of isolated plasmid from randomly selected 20 colonies using HiGene™ Plasmid Mini Prep kit (BIOFACT, Korea), and sequenced the inserts using primers pDONR 207 F and pDONR207 R (Macrogen, Korea) (Table 1). The rest of the cDNA library (900 μl) was divided into 300 μl aliquots, mixed with an equal volume of sterile 40 % glycerol and stored at –80 °C (Figure 1).

LR RECOMBINATION

Plasmids from randomly–selected colonies from the *B. tabaci* pDONR207 cDNA library were used for LR recombination with AltMV–*att*. All LR reacted colonies were recovered and purified AltMV–cDNA library fragments were transformed into *Agrobacterium tumefaciens* EHA105. In order to check for randomness and lack of redundancy we randomly selected 50 colonies and analyzed their plasmid sequences. To evaluate VIGS efficiency for AltMV and TRV containing the new Gateway system, we added β–ATPase and the five other genes used for test insertion which were amplified from *N. benthamiana* using the corresponding primers (Table 1). The genes selected for evaluation were Glutathione transferase (GSTU4 GenBank: JF915552.1), Ankyrin repeat–rich protein (ankyrin GenBank: GQ261740.1), bHLH2 transcription factor (bHLH2 GenBank: GQ859153.1) and

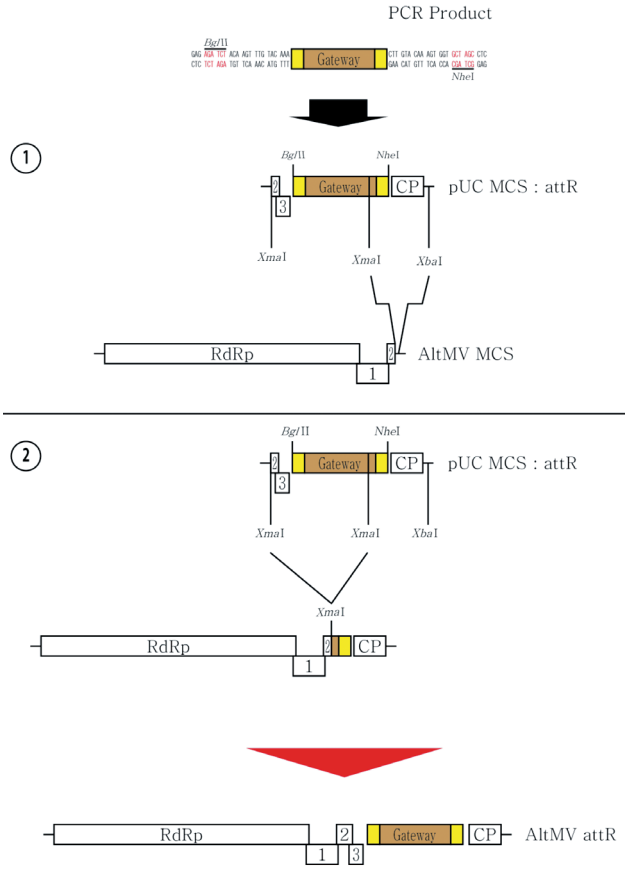

Fig. 1. AltMV *att*R construction process. Gateway *att*R site was amplified by primers containing 5' *Bgl*II and 3' *Nhe*I restrict enzyme site sequences (Table 1). This PCR product was inserted into pUC MCS (Lim *et al*., 2010a) vector using restriction enzymes *Bgl*II and *Nhe*I. ① *Xma*I and *Xba*I cut pUC–MCS product was ligated to *Xma*I and *Xba*I cut AltMV–MCS. Subsequently, ② the modified AltMV was cut by *Xma*I in order to insert the remainder of the Gateway att site (the *Xma*I–TGB2–3–Gateway–*Xma*I product), creating AltMV *att*R.

Myosin XI–K (XI–K GenBank: DQ875137.1) of *Nicotiana benthamiana*, and chickadee of *Bemisia tabaci*. Approximately 300 bp of each of the five genes were amplified by PCR, and equivalent amounts of each PCR product utilized in a multiplex Gateway LR reaction for insertion into the AltMV–*att* and TRV–*att* vectors to evaluate efficiency of the independent cloning of each insert. The AltMV–*att* and TRV–*att* β–ATPase constructs were used for agroinfiltration of *N. benthamiana* essentially as described (Lim *et al*., 2010a).. To evaluate the relative silencing efficiency of β–ATPase using each vector, quantitative real time qPCR was performed as described in Lim *et a*l. (2010a).

RESULTS

CONSTRUCTION OF ALTMV AND TRV VECTORS CONTAINING THE GATEWAY CLONING SYSTEM AND ITS EFFICIENCY

The recently developed AltMV vectors (Lim *et al*., 2010a) were developed for use with a wide variety of

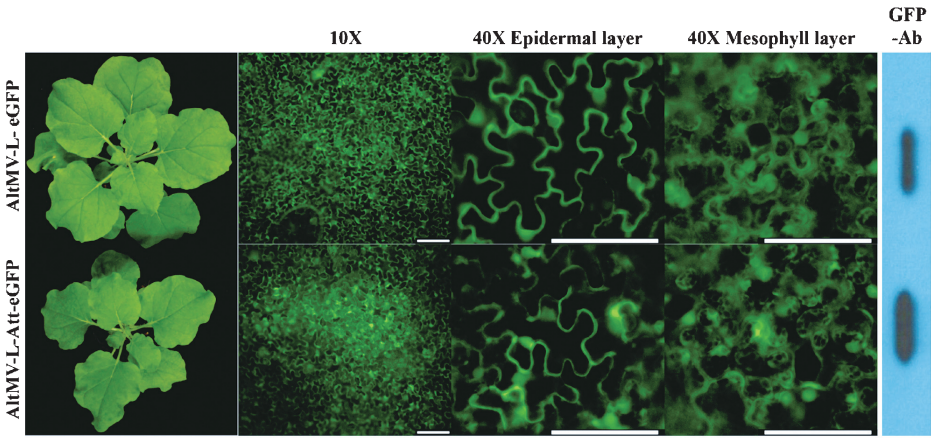

Fig. 2. Efficiency of expression of eGFP by AltMV–att was evaluated. Top: eGFP expression in control AltMV, in which eGFP was inserted to MCS; and bottom: eGFP inserted to att Gateway site in AltMV. GFP was measured by 480 nm fluorescence; bar indicates 50 um. Expression levels of eGFP production was also compared for each construct by western blot using GFP antibody (shown at right).

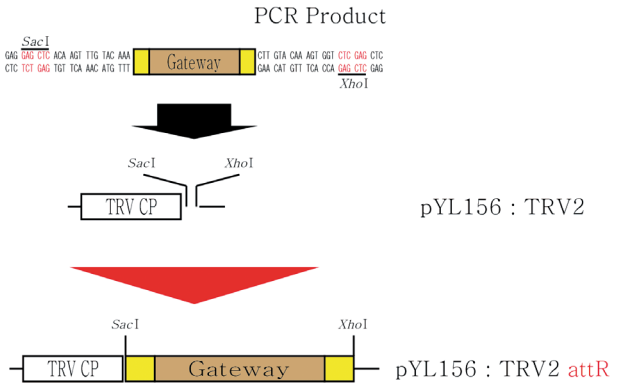

Fig. 2. TRV *att*R construction method. Gateway att site was amplified in a manner similar to creation of the AltMV–att vector using primers containing 5' *Sac*I and 3' *Xho*I restriction enzyme sites. PCR amplified Gateway cassette was inserted into *Sac*I and *Xho*I cut pYL156: TRV2 vector , creating TRV2 *att*R.

plant species. We have now modified the AltMV vectors with the Gateway system. Later, the modified vectors were used to demonstrate high throughput selection systems in appropriate hosts.

First of all, AltMV–L (protein expression vector) and AltMV–P (VIGS vector) were cleaved at *Xma*I and *Xba*I sites (Figure 1), and the 3' fragment substituted by an equivalent fragment including the Gateway system cassette/multiple cloning site (MCS) in place of the original MCS as described in Materials and Methods, yielding AltMV–L–*att* and AltMV–P–*att* respectively. The enhanced green fluorescent protein (eGFP) gene was separately cloned into AltMV–L–*att* and AltMV–P–*att* using the Gateway system; all colonies derived from the Gateway LR reaction contained the eGFP gene (data not shown). Expression levels were compared to those from the original AltMV–L and AltMV–P vectors containing eGFP (Lim *et al*., 2010a). There were no significant differences in symptoms, eGFP expression, or between

Table 2. Efficiency test of Gateway cloning

Cloning gene	Gateway virus vector	
	AltMV *att*R	TRV2 *att*R
GSTU4	11.11%	16.6%
ankyrin	25.92%	20.8%
bHLH2	33.33%	16.6%
XI–K	14.81%	29.2%
chickadee	14.81%	8.3%

protein and VIGS expression of the respective Gateway–modified and original AltMV–L and AltMV–P vectors (Figure 2).

In addition to cloning efficiency and expression of eGFP, we analyzed whether similar–sized PCR products were cloned with similar efficiencies. Five different PCR products of ca. 250 bp were amplified from *N. benthamiana*, and equivalent amounts of these PCR products utilized in a multiplex Gateway LR reaction for insertion into AltMV–P–*att*. Of 25 randomly–selected colonies sequenced, the ratio of each PCR product was 6:6:3:5:5 (Table 2). A TRV construct containing the Gateway *att* site (Figure 3) was similarly tested for random cloning efficiency; TRV–*att* showed a similar pattern of random insertion as in AltMV (Table 2). Therefore, we concluded that Gateway cloning could be used to efficiently clone libraries of similarly sized random fragments into the AltMV–P–*att* and TRV–*att* vectors without obvious bias, including size–fractionated sonicated cDNA (Figure 4).

To evaluate the relative efficiency of VIGS function, the same target gene construct was cloned in the *att* site of each vector. An approx. 250 bp fragment of chloroplast β ATPase was amplified, which was an adequate size for comparison of VIGS function between TRV and AltMV.

At 30 days post inoculation, plants showed different symptoms depending on reduction of chloroplast

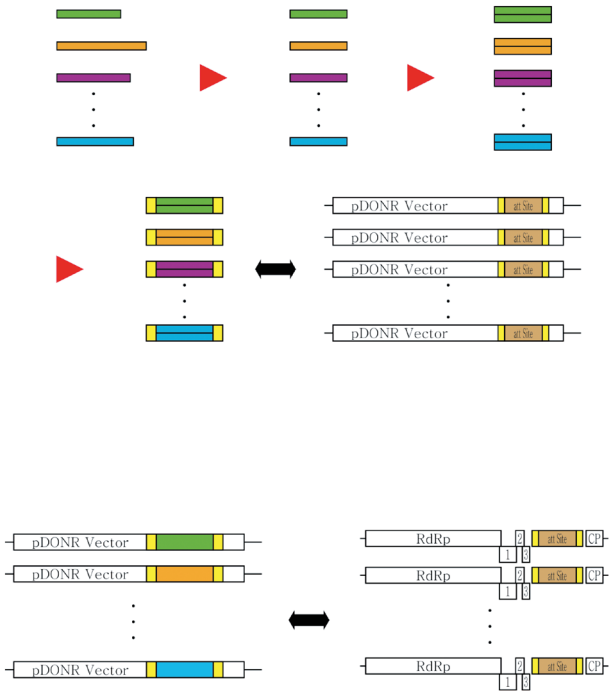

Fig. 4. Gateway cloning efficiency in cloning similarly sized various fragments into the AltMV–P–*att* vector. After similar size fragments of each gene were amplified, each PCR product was inserted into pDONR vector by BP recombination. An equal concentration of each gene product inserted in pDONR vector was inserted into AltMV by LR recombination.

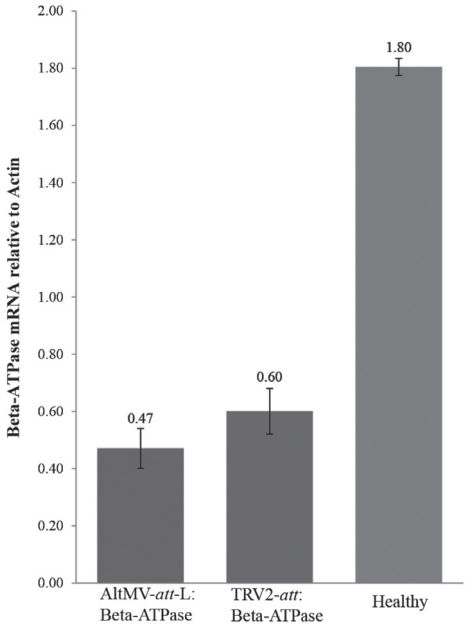

Fig. 5. Comparison of *Nicotiana benthamiana* chloroplast β ATPase silencing efficiency using AltMV and TRV vectors. Five plants were agro–infiltrated with each of AltMV–*att*–L and TRV2–*att* including a Chloroplast β ATPase RNAi sequence. The β ATPase mRNA expression level was measured by real time qPCR. *N benthamiana* β ATPase was silenced by AltMV–*att*–L and TRV2–*att* respectively showed a 3.8 and 3 fold reduction in β ATPase mRNA expression level as compared with healthy *N. benthamiana*.

β–ATPase expression induced by each of the viral vectors; AltMV–*att*[β–ATPase] induced an increased area of chlorosis compared to TRV–*att*[β–ATPase], and to wild type AltMV or TRV respectively, and a greater reduction of β–ATPase expression (Figure 5). However, the chlorotic phenotype induced by reduction of chloroplast β–ATPase expression was not as dramatic as AltMV– or TRV–mediated VIGS of phytoene desaturase. However, reverse–transcriptase quantitative PCR indicated that chloroplast β ATPase mRNA accululation in AltMV chloroplast β–ATPase RNAi–infected plants was lower than that in TRV chloroplast β–ATPase RNAi–infected plants, and about four fold lower compared to healthy.

PREPARATION OF A *BEMISIA TABACI* 250–350 BP cDNA LIBRARY IN A GATEWAY SYSTEM MODIFIED VECTOR

A *B. tabaci* cDNA library was prepared as described at M & M. However, as we do not need cDNA representing the entire mRNA we sonicated cDNA and added adapters in order to produce amplified random fragments of suitable size (between 250–350bp). The size of fragments was dependent on sonication time, and one sec sonication time produced a relatively uniform population of fragments of the desired size.

Sonicated DNA fragments were amplified after addition of *att* adapter sequences (Figure 6) and cloned directly to AltMV–*att*–P. A total of 1.2×10^5 colonies were recovered in *E. coli*, and 1.4×10^3 colonies in *A. tumefaciens*. To examine the redundancy of the library, we selected 50 *A. tumefaciens* colonies and analyzed sequence of each AltMV–*att* clone (Table 3). Sequence data revealed that no overlapping sequences and no contigs existed among these randomly selected clones. Most of the sequences were identified as derived from *B. tabaci*, but unannotated sequence were also common. The insert sizes ranged from 150 bp to 70bp, with 90% between 200–300 bp from 50 colonies.

DISCUSSION

RNAi is a highly effective method of reverse genetics to examine and identify gene function. VIGS is a method that has been used to apply RNAi to a range of model and crop plant species. The most widely used VIGS vectors to date have been TRV and BSMV. For each of these vectors, ligation independent cloning (LIC) strategies have been developed that are described as high throughput systems (Dong *et al.*, 2007; Yuan *et al.*, 2011). However, as described in these reports, the LIC strategy utilized tagged PCR primers specifically designed for amplification and cloning of each target gene. In contrast, the approach we describe here allows for the preparation of random cDNA libraries (or in principle, random genomic libraries) of short fragments of suitable length for inducing gene silencing, incorporating the Gateway cloning system. These random cDNA libraries can then be efficiently transferred to the VIGS vector without obvious bias, and the VIGS vector cDNA library transformed into *Agrobacterium* for delivery of individual clones to

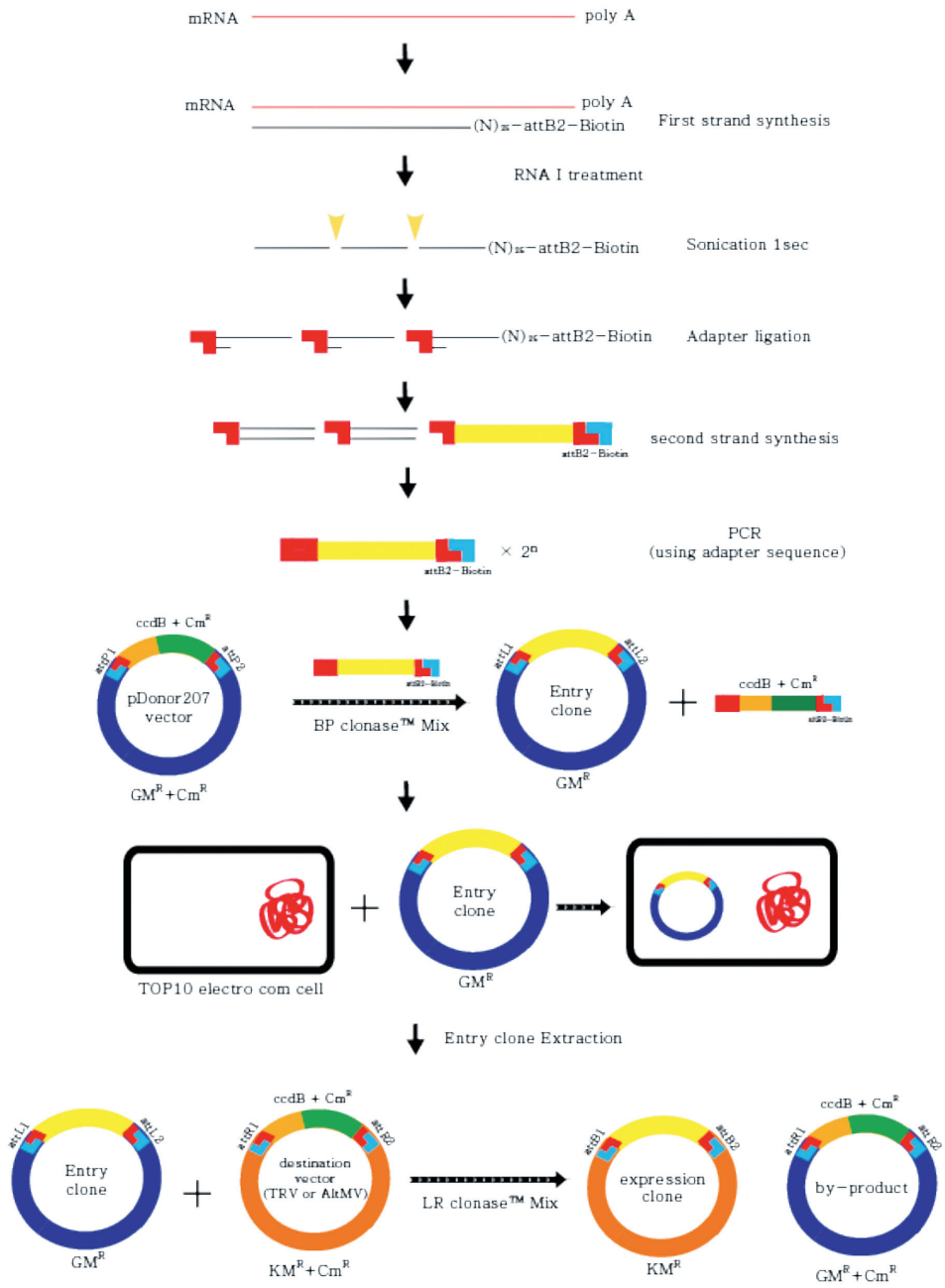

Fig. 6. *Bemisia tabaci* cDNA library process using gateway system. After isolating *B. tabaci* mRNA, first strand cDNA was synthesized and sonicated for 1 sec. After ligating adapters, second strand cDNA was synthesized, and amplified using *att* adapter primers. The PCR product was inserted into pDONR 207 vector by Gateway BP recombination. Entry clones were selected on Gentamycin (GM^R) plates, and used to transform One shot® TOP 10 electrocompetent cells. Plasmids extracted from about 1.75×10^6 colonies of *B. tabaci* cDNA were inserted into TRV2–*att*R or AltMV–*att*R destination vectors by Gateway LR recombination. Expression vectors were selected on Kanamycin(KM^R) plates. (GM^R: Gentamycin resistance, Cm^R: Chloramphenicol resistance, KM^R: Kanamycin resistance)

the desired plant species by agroinfiltration. Although as described here the cDNA library was initially transformed into *E. coli*, the VIGS vector library could in principle be transformed directly into *Agrobacterium*. However, the efficiency of *Agrobacterium* transformation is typically far lower than of *E. coli*, and a significant loss of cDNA library coverage would be expected.

We demonstrated this approach by modifying a versatile AltMV vector (Lim *et al.*, 2010a) with the Gateway cloning system, and subsequent insertion into the Gateway–modified VIGS vector of a random cDNA library derived from the silverleaf whitefly (*B. tabaci*). *Bemisia tabaci* is a significant economic pest in its own right, but has gained even greater significance as a vector of many geminiviruses (e.g. Bedford *et al.*, 1994) and criniviruses (e.g. Tian *et al.*, 1999), which cause even greater crop losses. Here we have shown that the approach we have used resulted in creation of a diverse cDNA library of 250–350 bp fragments from *B. tabaci*, and that we have been able to rapidly screen large numbers of VIGS clones

Table 3. EST Blast search data of randomly–selected 50 colonies for confirm redundancy

No.	EST blast search	size (bp)	Accession number
No.1	U_U–R_Forward–3491.b1 *Bemisia tabaci* forward SSH library *Bemisia tabaci* cDNA, mRNA sequence	231	JK485896.1
No.2	unknown	101	unknown
No.3	unknown	771	unknown
No.4	TOMOV–BT006_E08 Whitefly *Bemisia tabaci*(reared on TOMOV infected plants) cDNA library *Bemisia tabaci* cDNA5', mRNA sequence.	95	EE599425.1
No.5	FP919033 *Acyrthosiphon pisum* whole insect males *Acyrthosiphon pisum* cDNA clone ACI0AAF8YK08 5', mRNA sequence.	139	FP919033.1
No.6	U_U–U_Forward–3830.b1 *Bemisia tabaci* forward SSH library *Bemisia tabaci* cDNA, mRNA sequence.	593	JK486234.1
No.7	BT–TYLCV–017–1–A5–T3_A05 Whitefly *Bemisia tabaci* (reared on TYLCV infected plants) cDNA library *Bemisia tabaci* cDNA 5', mRNA sequence.	364	EE600339.1
No.8	unknown	255	unknown
No.9	BT–TOMOV–017–1–A10–T3_A10 Whitefly *Bemisia tabaci* (reared on TOMOV infected plants) cDNA library *Bemisia tabaci* cDNA 5', mRNA sequence.	390	EE597788.1
No.10	HBT004_H01_T3_015 Whitefly *Bemisia tabaci* (non–viruliferous)cDNA library *Bemisia tabaci* cDNA5',mRNA sequence.	342	EE597198.1
No.11	2_Reverse–4323 *Bemisia tabaci* reverse SSH library *Bemisia tabaci* cDNA, mRNA sequence.	300	JK487193.1
No.12	unknown	316	unknown
No.13	BT–TYLCV–059–1–F4–T3_F04 Whitefly *Bemisia tabaci* (reared on TYLCV infected plants) cDNA library *Bemisia tabac*i cDNA5', mRNA sequence.	203	EE602025.1
No.14	BT_TYLCV003_A09 Whitefly *Bemisia tabaci* (reared on TYLCV infected plants) cDNA library *Bemisia tabaci* cDNA 5', mRNA sequence.	64	EE600011.1
No.15	unknown	114	unknown
No.16	ID0AAA3BF12FM1 ApMS *Acyrthosiphon pisum* cDNA clone ID0AAA3BF12 5', mRNA sequence	653	CN761972.1
No.17	BT_TYLCV003_A09 Whitefly *Bemisia tabaci* (reared on TYLCV infected plants)cDNA library *Bemisia tabaci* cDNA 5', mRNA sequence.	177	EE600011.1
No.18	unknown	84	unknown
No.19	Sequencing fail		Sequencing fail
No.20	unknown	79	unknown
No.21	U_U–I_Forward–2627.b1 *Bemisia tabaci* forward SSH library *Bemisia tabaci* cDNA, mRNA sequence.	314	JK485039.1
No.22	unknown	108	unknown
No.23	unknown	296	unknown
No.24	BT–HINST–001–1–H11–T3_H11 Whitefly *Bemisia tabaci* instar (crawler to pupae) cDNA library *Bemisia tabaci* cDNA 5', mRNA sequence.	206	EE602760.1
No.25	U_U–I_Forward–3278.b1 *Bemisia tabaci* forward SSH library *Bemisia tabaci* cDNA, mRNA sequence.	322	JK485683.1
No.26	BT–HINST–004–1–H3–T3_H03 Whitefly *Bemisia tabaci* instar (crawler to pupae) cDNA library *Bemisia tabaci* cDNA 5', mRNA sequence.	215	EE602910.1
No.27	unknown	156	unknown
No.28	unknown	267	unknown
No.29	unknown	269	unknown
No.30	unknown	734	unknown
No.31	CATF10874.fwd CATF *Artemisia annua*, Tanzanian, from Sandeman Seeds, 1–2kb *Artemisia annua* cDNA clone CATF10874 5', mRNA sequence	341	EY058051.1
No.32	BT–HINST–021–1–A9–T3_A09 Whitefly *Bemisia tabaci* instar (crawler to pupae) cDNA library *Bemisia tabaci* cDNA5',mRNA sequence.	135	EE603681.1

No.	EST blast search	size (bp)	Accession number
No.33	NACBU27TF *Aedes aegypti* infected with Brugia Malayi *Aedes aegypti* cDNA clone NACBU27,mRNA sequence.	501	DV366139.1
No.34	BT–TYLCV–018–1–H5–T3_H05 Whitefly *Bemisia tabaci* (reared on TYLCV infected plants) cDNA library *Bemisia tabaci* cDNA 5', mRNA sequence	155	EE600421.1
No.35	unknown	238	unknown
No.36	BT–TOMOV–014–1–H12–T3_H12 Whitefly *Bemisia tabaci* (reared on TOMOV infected plants) cDNA library *Bemisia tabaci* cDNA 5', mRNA sequence	162	FM143549.1
No.37	BT–TYLCV–048–1–B11–T3_B11 Whitefly *Bemisia tabaci*(reared on TYLCV infected plants) cDNA library *Bemisia tabaci* cDNA5',mRNA sequence.	203	EE601565.1
No.38	unknown	143	unknown
No.39	BT–TYLCV–059–1–F4–T3_F04 Whitefly *Bemisia tabaci*(reared on TYLCV infected plants) cDNA library *Bemisia tabaci* cDNA 5', mRNA sequence.	385	EE602025.1
No.40	BT–TYLCV–020–1–D4–T3_D04 Whitefly *Bemisia tabaci* (reared on TYLCV infected plants)cDNA library *Bemisia tabaci* cDNA5',mRNA sequence.	202	EE600496.1
No.41	3_Reverse–4493 *Bemisia tabaci* reverse SSH library *Bemisia tabaci* cDNA, mRNA sequence.	293	JK487347.1
No.42	BT–TYLCV–021–1–A7–T3_A07 Whitefly *Bemisia tabaci* (reared on TYLCV infected plants) cDNA library *Bemisia tabaci* cDNA 5', mRNA	573	EE600529.1
No.43	BT–TYLCV–054–1–E11–T3_E11 Whitefly *Bemisia tabaci* (reared on TYLCV infected plants) cDNA library *Bemisia tabaci* cDNA 5', mRNA sequence.	227	EE674663.1
No.44	unknown	239	unknown
No.45	Sequencing fail.		Sequencing fail
No.46	unknown	573	unknown
No.47	BT–TYLCV–060–1–E4–T3_E04 Whitefly *Bemisia tabaci*(reared on TYLCV infected plants)cDNA library *Bemisia tabaci* cDNA5',mRNA sequence.	137	EE602067.1
No.48	unknown	500	unknown
No.49	unknown	265	unknown
No.50	A_Forward–1888 *Bemisia tabaci* forward SSH library *Bemisia tabaci* cDNA, mRNA sequence.	230	JK486690.1

by agroinfiltration of individual plants with *Agrobacterium* clones derived from this library.

Delivery of siRNAs to a number of different insect species by a variety of methods has been utilized to identify gene function, and some constructs demonstrated to affect insect growth and fecundity. Injected dsRNA (Yang *et al*., 2000) or synthetic siRNAs (Boutla *et al*., 2003) have been shown to induce silencing in *Drosophila* embryos. Injected siRNA has also been used for testing gene function in other insects; RNA mediated gene knockdown demonstrated down–regulation of the C002 gene in salivary glands of *Acyrthosiphon pisum* (Mutti *et al*., 2006). In addition, Chickadee and HSP genes were tested for down–regulation of these genes in whitefly (Ghanim *et al*., 2007; Lü and Wan, 2011). siRNA injection methods were used for these purposes, but the siRNA injection method is not suitable for mass application in order to control insects, or for high–throughput selection of effective RNAi constructs for insect control.

Plant–mediated RNAi control through ingestion by the insects from transfected or stably transgenic plants has been demonstrated; examples include control of larvae of the cotton bollworm, *Helicoverpa armigera* (Mao *et al*., 2007) and the Western corn rootworm, *Diabrotica virgifera vergifera* (Baum *et al*., 2007), as well as silencing of midgut genes of the brown plant hopper, *Nilaparvata lugens* (Zha *et al*., 2011). More recently aphid genes have been shown to be silenced following feeding of *Myzus persicae* on *N. benthamiana* transiently expressing dsRNA constructs following agroinfiltration, or stable transgenic *Arabidopsis*. The RNAi targets were genes known to be expressed in the aphid gut or salivary glands, and reductions in aphid fecundity were observed (Pitino *et al*., 2011). Either transgenic plant or agroinfiltration expression of RNAi against *M. persicae* effectors COO2, PIntO1 and PIntO2 reduced fecundity, while over–expression of the same effectors in transgenic plants increased fecundity (Pitino and Hogenhout, 2013). RNAi constructs of a serine protease gene of *M. persicae* expressed in transgenic *Arabidopsis* resulted in a decline in serine protease activity in the gut of aphids feeding on these plants, leading to a decline in fecundity (Bhatia *et al*., 2012). In another study, a comparison of dsRNA injection versus feeding of dsRNA mixed with an artificial diet demonstrated differences in localization and effectiveness of silencing of the cathepsin–L gene in *A. pisum*; feeding resulted in far more effective silencing in the gut and

alteration of specific gut epithelial cells, allowing better characterization of a tissue–specific role of the gene. In contrast, injection was more effective in the head and carcass and suggested a novel role for cathepsin–L in molting (Sapountzis *et al*., 2014).

RNAi directed against plant genes has also been shown to influence susceptibility to insect pests; Bosch *et al*. (2014) down–regulated tomato OPR3, a key enzyme in the jasmonic acid (JA) pathway, and demonstrated that the transgenic plants deficient for JA–related defenses were more attractive to feeding and oviposition by *Manduca sexta*.

Such transgenic approaches will likely be necessary for application of effective RNAi constructs at the field level; however, testing of multiple genes and gene constructs by production of transgenic plants is slow and laborious, whereas a VIGS approach offers a far faster method for determination of gene function (Scofield and Nelson, 2009). We have therefore developed the wide host range virus AltMV vector for high–throughput study of gene function, and have selected control of the whitefly *B. tabaci* as the system in which to demonstrate the capabilities of the system through feeding whitefly on plants infected with AltMV expressing *B. tabaci* RNAi constructs inserted directly from a cDNA library. The *B. tabaci* first strand cDNA was sonicated to produce fragments primarily within the preferred 250–350 bp range, and the cDNA library inserted directly to the AltMV–P–att vector modified with the Gateway system. Individual *Agrobacterium* clones from this cDNA library were then utilized to agroinfiltrate single tomato plants to establish RNAi by VIGS, and exposed to a *B. tabaci* population in order to observe effects on whitely mortality and fecundity. Our newly developed high–throughput screening system could thereby be utilized to identify potential RNAi constructs for control of this economically important insect with no *a priori* selection of constructs, and with a minimum of time between preparations of a random cDNA library to initial screening in planta. The main limitation to screening large numbers of potential RNAi constructs thus becomes the space available for raising an adequate plant population, and maintenance of a population of the insect pest with which to challenge the plant population. Agroinfiltration of single or replicate plants with individual members of the AltMV–P–*att* cDNA library requires only a few minutes per construct. Visual observation over the days following exposure to the whitefly population allows identification of RNAi constructs with apparent efficacy for further testing, sequencing of the selected constructs to identify the genes conferring the desired phenotype, and design of primers for qRT–PCR to determine the levels of knockdown of gene expression within the whitefly. The most efficacious constructs can then be directly transferred to an appropriate plant transformation vector utilizing the Gateway system, and used to transform plants for further evaluation on a larger scale and under various environmental conditions.

ACKNOWLEDGEMENT

This research was supported by Agricultural Biotechnology Development Program (Project No. 112018–3) and Technology Commercialization Support Program (Project No. 113044–3), Ministry of Agriculture, Food and Rural Affairs.

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