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

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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. 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. 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

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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). Reiner 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 attR1_F_BglII and attR2_R_Nhel (Table 1). Then the PCR product was inserted into the pUC MCS vector (Lim et al., 2010a) digested with BglII and Nhel (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 : attR were each digested with XmaI and XhoI. The ‘3’ part of pUC MCS : attR was inserted into AltMV MCS, followed by the remaining XmaI fragment, creating AltMV–attR. After ligation and sequencing to confirm the correct structure, AltMV–attR 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’ SacI and 3’ XhoI recognitions sequences was amplified essentially as described above using primers attR1_F_SacI and attR2_R_XhoI (Table 1). This cassette was inserted into pYL156:TRV2 between the SacI and XhoI sites. As with the AltMV–attR Gateway cloning vector, pYL156:TRV–attR 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
Developing an AltMV Vector for Efficient Cloning to Screen Gene Function

(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\textsuperscript{R} MAG mRNA isolation kit (Invitrogen), following the manufacturer's protocol. Purified mRNA was used to synthesize first strand cDNA using random primer Biotin–att\textsubscript{B2}–(N)\textsubscript{6} (Table 1) and SuperScript\textsuperscript{R} Full length cDNA Library Construction Kit \textsuperscript{II} protocol with some modifications (see below). The primer was designed to include the att\textsubscript{B2} sequence, with (N)\textsubscript{6} added in order to allow the primer bind to mRNA at random locations. Then the first strand cDNA was sonicated with a Vcx 750 Watt ultrasonic processor (Sonics & Materials) for 1

Table 1. Primers used in this study.

<table>
<thead>
<tr>
<th>Clone</th>
<th>5’–Oligo</th>
<th>5’–Oligo sequence</th>
<th>3’–oligo</th>
<th>3’–Oligo sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AltMV vector</td>
<td>attR1_F_BglII</td>
<td>ACAAGTTTGTACAAAAAAGCTGAAC</td>
<td>attR2_R_Nhel</td>
<td>ACCACTTTGTACAAGAAAGCTG</td>
</tr>
<tr>
<td>TRV vector</td>
<td>attR1_F_SacI</td>
<td>GAGGAGCTCAAGATTTGTACAAAAAAGCTG AAC</td>
<td>attR2_R_Xhol</td>
<td>GAGCTCGAGACCACCTTTGTACAAGAAAGCTG AAAGCTG</td>
</tr>
<tr>
<td>Primers used in cloning vector construction</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primers used in cDNA library construction</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primers used in BP recombinant assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primers used in Gateway Cloning Efficiency Test assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(B. tabaci)

Chickadee Chickadee F TGGCAAGAGTGTTTTCAAAATGA Chickadee R GGGGACCACTTTGTACAAGAAAGCTG |

LR_insert sequence analyze

attR2 R ACCACTTTGTACAAGAAAGCTG |

Primers used in QRT–PCR

N. benthamiana Actin

Actin_F | TGGCAAGAGTGTTTTCAAAATGA |

Actin_R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana beta ATPase

beta_F | TGGCAAGAGTGTTTTCAAAATGA |

beta_R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana XI–K F

XI–K F | TGGCAAGAGTGTTTTCAAAATGA |

XI–K R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana Ankyrin

Ankyrin_F | TGGCAAGAGTGTTTTCAAAATGA |

Ankyrin_R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana bHLH2

bHLH2_F | TGGCAAGAGTGTTTTCAAAATGA |

bHLH2_R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana GSTU4

GSTU4_F | TGGCAAGAGTGTTTTCAAAATGA |

GSTU4_R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana miniGSTU4

miniGSTU4_F | TGGCAAGAGTGTTTTCAAAATGA |

miniGSTU4_R | GGGGACCACTTTGTACAAGAAAGCTG |

Primers used in Gateway Cloning Efficiency Test assay

N. benthamiana GSTU4

GSTU4_F | TGGCAAGAGTGTTTTCAAAATGA |

GSTU4_R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana Ankyrin

Ankyrin_F | TGGCAAGAGTGTTTTCAAAATGA |

Ankyrin_R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana bHLH2

bHLH2_F | TGGCAAGAGTGTTTTCAAAATGA |

bHLH2_R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana XI–K F

XI–K F | TGGCAAGAGTGTTTTCAAAATGA |

XI–K R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana Ankyrin

Ankyrin_F | TGGCAAGAGTGTTTTCAAAATGA |

Ankyrin_R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana bHLH2

bHLH2_F | TGGCAAGAGTGTTTTCAAAATGA |

bHLH2_R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana XI–K F

XI–K F | TGGCAAGAGTGTTTTCAAAATGA |

XI–K R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana Ankyrin

Ankyrin_F | TGGCAAGAGTGTTTTCAAAATGA |

Ankyrin_R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana bHLH2

bHLH2_F | TGGCAAGAGTGTTTTCAAAATGA |

bHLH2_R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana XI–K F

XI–K F | TGGCAAGAGTGTTTTCAAAATGA |

XI–K R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana Ankyrin

Ankyrin_F | TGGCAAGAGTGTTTTCAAAATGA |

Ankyrin_R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana bHLH2

bHLH2_F | TGGCAAGAGTGTTTTCAAAATGA |

bHLH2_R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana XI–K F

XI–K F | TGGCAAGAGTGTTTTCAAAATGA |

XI–K R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana Ankyrin

Ankyrin_F | TGGCAAGAGTGTTTTCAAAATGA |

Ankyrin_R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana bHLH2

bHLH2_F | TGGCAAGAGTGTTTTCAAAATGA |

bHLH2_R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana XI–K F

XI–K F | TGGCAAGAGTGTTTTCAAAATGA |

XI–K R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana Ankyrin

Ankyrin_F | TGGCAAGAGTGTTTTCAAAATGA |

Ankyrin_R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana bHLH2

bHLH2_F | TGGCAAGAGTGTTTTCAAAATGA |

bHLH2_R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana XI–K F

XI–K F | TGGCAAGAGTGTTTTCAAAATGA |

XI–K R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana Ankyrin

Ankyrin_F | TGGCAAGAGTGTTTTCAAAATGA |

Ankyrin_R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana bHLH2

bHLH2_F | TGGCAAGAGTGTTTTCAAAATGA |

bHLH2_R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana XI–K F

XI–K F | TGGCAAGAGTGTTTTCAAAATGA |

XI–K R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana Ankyrin

Ankyrin_F | TGGCAAGAGTGTTTTCAAAATGA |

Ankyrin_R | GGGGACCACTTTGTACAAGAAAGCTG |

N. benthamiana bHLH2

bHLH2_F | TGGCAAGAGTGTTTTCAAAATGA |

bHLH2_R | GGGGACCACTTTGTACAAGAAAGCTG |
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' attB1 adapter (attB1–adapter–U annealed with attB1–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 attB–flanked DNA was amplified with a mixture of Biotin–attB2–(N)6 and Biotin–attB2–(N), with attB1–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 attB–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 µl 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 10−3 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 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 al. (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
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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 XmaI and XbaI 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 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 were 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

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**Table 2. Efficiency test of Gateway cloning**

<table>
<thead>
<tr>
<th>Cloning gene</th>
<th>AltMV attR</th>
<th>TRV2 attR</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTU4</td>
<td>11.11%</td>
<td>16.6%</td>
</tr>
<tr>
<td>ankyrin</td>
<td>25.92%</td>
<td>20.8%</td>
</tr>
<tr>
<td>bHLH2</td>
<td>33.33%</td>
<td>16.6%</td>
</tr>
<tr>
<td>XI–K</td>
<td>14.81%</td>
<td>29.2%</td>
</tr>
<tr>
<td>chickadee</td>
<td>14.81%</td>
<td>8.3%</td>
</tr>
</tbody>
</table>

---

**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 attR construction method. Gateway att site was amplified in a manner similar to creation of the AltMV–att vector using primers containing 5’ SacI and 3’ XhoI restriction enzyme sites. PCR amplified Gateway cassette was inserted into SacI and XhoI cut pYL156: TRV2 vector, creating TRV2 attR.
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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 accumulation 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 1.2×10⁵ colonies were recovered in E. coli, and 1.4×10³ 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...
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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 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.

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

<table>
<thead>
<tr>
<th>No.</th>
<th>EST blast search</th>
<th>size (bp)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.1</td>
<td>U_U–R_Forward–3491.b1 <em>Bemisia tabaci</em> forward SSH library <em>Bemisia tabaci</em> cDNA, mRNA sequence</td>
<td>231</td>
<td>JK485896.1</td>
</tr>
<tr>
<td>No.2</td>
<td>unknown</td>
<td>101</td>
<td>unknown</td>
</tr>
<tr>
<td>No.3</td>
<td>unknown</td>
<td>771</td>
<td>unknown</td>
</tr>
<tr>
<td>No.4</td>
<td>TOMOV–BT006_E08 <em>Whitefly Bemisia tabaci</em> (reared on TOMOV infected plants) cDNA library <em>Bemisia tabaci</em> cDNA5', mRNA sequence.</td>
<td>95</td>
<td>EE599425.1</td>
</tr>
<tr>
<td>No.5</td>
<td>FP919033 <em>Acyrthosiphon pisum</em> whole insect males <em>Acyrthosiphon pisum</em> cDNA clone AC01AA85K08 5', mRNA sequence.</td>
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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 virgifera (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

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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 carcas 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 whitefly 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 a priori selection of constructs, and with a minimum of time between preparations of a random cDNA library to initial screening in plants. 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

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