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A High Throughput Soybean Gene Identification System Developed using *Soybean Yellow Common Mosaic Virus* (SYCMV)

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Soybean yellow common mosaic virus (SYCMV) was recently reported from Korea, and a subsequent survey of soybean fields found that SYCMV, *Soybean yellow mottle mosaic virus* (SYMMV), and *Soybean mosaic* virus (SMV) infections were widespread. SYCMV has recently been developed into a Virus Inducing Gene Silencing (VIGS) vector for use as a reverse genetics tool for soybean, and here we report a modified SYCMV VIGS vector containing a new restriction enzyme site in the 3' non–coding region into which we inserted the Gateway system. Ultrasonically generated c.300 bp random fragments of *Glycine max* cDNA were inserted into the SYCMV VIGS vector, and individual colonies containing *G. max* cDNA were inoculated to cultivar Williams 82. We monitored the phenotype of inoculated soybean, and selected obvious visible phenotypes caused by SYCMV–induced gene silencing which could enable annotation of gene function of unknown gene fragments inserted into SYCMV. Here, we describe development of a high–throughput SYCMV VIGS vector for gene function identification in soybean.

Key words: *Soybean yellow common mosaic virus*, Soybean cDNA library, Virus induced gene silencing, Gateway cloning system, Reverse genetics

INTRODUCTION

There is increasing reliance upon dietary protein produced by plants, such as soybean, which is one of the main crops to supply protein source. Soybean also contains a high amount of isoflavone phytoestrogen, reported to inhibit tumor growth (Harper *et al*., 1996; Folkman and Shing, 1992) and could thus be used for protective medicine as well as a main protein resource. In Korea the production of soybean has increased annually because of increased consumer demand. However soybean virus disease is a major problem because a large number of farmers plant self–saved soybean seed. Therefore, a nationwide survey was recently performed to identify the main viruses infecting soybean in subsistence soybean fields (Cho *et al*., 2013).

This nationwide survey indicated that *Soybean mosaic virus* (SMV), *Soybean yellow mottle mosaic virus* (SYMMV) and Soybean yellow common mosaic virus (SYCMV) were most commonly found in the field, and Soybean common mosaic virus (SCMV) was also detected (Cho *et al*., 2013).

SMV is a member of the genus *Potyvirus* which has a strong silencing suppressor, and is highly seed transmitted (Domier *et al*., 2011). SYMMV is a new member of the genus *Carmovirus* in the family *Tombusviridae*, and was reported in Korea to infect only soybean (Nam *et al*., 2009). The sequence of SYCMV shows very low nucleotide sequence identity (25.4–34.3%) with those of members of the genus *Sobemovirus* (Nam *et al*., 2012). The newly reported SCMV is also in the genus *Potyvirus*.

Many plant viruses induce an RNA–mediated plant defense mechanism involving gene silencing, which has been exploited for plant reverse genetics by utilization of virus–induced gene silencing (VIGS) to determine the functions of host genes (Baulcombe, 1999, 2004; Godge *et al*., 2008). Among the best known VIGS vectors for identifying host gene function are *Tobacco rattle virus* (TRV) for dicotyledonous plants (Burch–Smith *et al*., 2004), and *Barley stripe mosaic virus* (BSMV) for gramineaceous species (Scofield and Nelson, 2009; Cakir *et al*., 2010). The replication of the VIGS vector generates double–stranded RNA, resulting in expression of viral–induced siRNA (also known as RNAi) corresponding to both the viral genomic RNA and the inserted host gene mRNA target sequences; VIGS knock–down of tar-

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get mRNA leads to a phenotype distinct from the wild type (Baulcombe, 1999, 2004) that is most easily detected if the phenotype is visible. Because VIGS can be implemented much more rapidly if a suitable VIGS vector is available, use of VIGS saves considerable time and expense compared to production of transgenic plants for examination of gene function (Scofield and Nelson, 2009). Many plant viruses are able to overcome the host plant defense mechanisms through expression of suppressors of RNA silencing, such as the HC–Pro of *Potyvirus*es, 2b protein of cucumoviruses, and TGB1 of potexviruses and hordeiviruses (Brigneti *et al*., 1998; Voinnet *et al*., 2000). Modification or selection for weaker viral silencing suppressor function may increase the effectiveness of a virus as a VIGS vector (Lim *et al*., 2010 [Virology]), and allow development of new viral vectors for specific crops.

SMV, the most common virus affecting soybean, is a *Potyvirus* with an efficient HC–Pro suppressor of RNA silencing, and thus unsuitable for use as a VIGS vector. Although Bean pod mottle virus (BPMV) has been utilized as a VIGS vector (Zhang and Ghabrial, 2006), BPMV has not been reported outside North America, and is thus not appropriate for use in Korea. However, the recently described SYCMV (Nam *et al*., 2012) produces relative mild symptoms and has a relatively inefficient suppressor of RNA silencing, making it highly suitable as a VIGS vector. We report here modification of an infectious clone of SYCMV with a multiple cloning site (MCS) in the 3' non–coding region, into which we have introduced the Gateway cloning system, and demonstrate the potential for high–throughput insertion of constructs for VIGS and gene function identification in soybean.

MATERIALS AND METHODS

TOTAL RNA EXTRACTION AND mRNA ISOLATION FROM SOYBEAN *GLYCINE MAX*

Total RNA was extracted from leaves of four week–old *Glycine max* using Trizol reagent (MRC) and mRNA isolation was performed with FastTrack® MAG mRNA isolation Kit (invitrogen) following the manufacturer's recommended protocol, with mRNA eluted in RNase–free water.

CONSTRUCTION OF AN RNAi cDNA LIBRARY USING THE GATEWAY SYSTEM

Purified mRNA was directly used for first strand cDNA synthesis using 3' primer Biotin–*att*B2–$(N)_{25}$ (Table 1), and the RNA digested with RNase I. The resulting first strand cDNA was sonicated for 1 second to produce small size fragment (from 250 bp to 500 bp) with a Vcx 750 Watt ultrasonic probe. The double stranded 5' *att*B1–adapter oligomers (Table 1) used for Gateway system cloning were annealed to each other and then ligated to the ends of the 15,000 μg cDNA fragments using T4 DNA ligase (Figure 1). After 5' *att*B1–adapter arms were attached to the cDNA fragments, the second strands were synthesized by extension using Taq™ DNA polymerase. The double–stranded fragments were annealed with *att*B2 to be attached at the 3'ends of the DNA fragment, and finally amplified using a mixture of primers *att*B2–$(N)_{25}$ and *att*B2–$(N)_6$ to generate suitable PCR fragment size to construct RNAi cDNA library (Table 1).

Table 1. Oligomers used in this Study

Oligomer	Sequence	Feature
Oligomers used in synthesis of cDNA fragments with *att* site		
5' *att*B1–adapter	5'–TCGTCGGGG**ACAACTTTGTACAAAAAAGTTGG**– 3' 3'– CCCC**TGTTGAAACATGTTTTTTCAACC**p– 5'	5' *att*B1–adapter
Biotine–*att*B2–$(N)_{25}$	5'–Biotin–GGGGACAACTTTGTACAAGAAAGTTGGG(N)25–3'	Using first strand synthesis
Oligomers used in amplifying cDNA		
5' *att*B1–oligo	5'–TCGTCGGGGACAACTTTGTACAAAAAAGTTGG–3'	5'–Oligo
Biotine–*att*B2–$(N)_6$	5'–Biotin–GGGGACAACTTTGTACAAGAAAGTTGGG(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 Agrobacteria		
pSYCMV *att* B1	5'–GAT GCT AAC ATC GCG ACT C–3'	5'–Oligo
pSYCMV *att* B2	5'–CAT TTG GAT TAC GCT CCA TTT C–3'	3'–Oligo
Oligomers used in synthesis of multiple cloning site		
BsrGI MCS3	5'– **GTACA**GAGGGGCCCGAGATTTAAATGAGACTAGTGAGAGGCCTGAG**T**–3' 3'– **T**CTCCCCGGGCTCTAAATTTACTCTGATCACTCTCCGGACTC**ACATG** –5'	Two *BsrGI* MCS3 oligomers were annealed to each other, ***BsrGI***
Oligomers used in Gateway cloning adapter including restriction enzyme site.		
StuI att site F	5'–GAG **AGG CCT** CAC AAG TTT GTA CAA AAA AGC TG–3'	5'–Oligo, ***StuI***
SpeI att site R	5'–GAG **ACT AGT** CAA CCA CTT TGT ACA AGA AGG C–3'	3'–Oligo, ***SpeI***

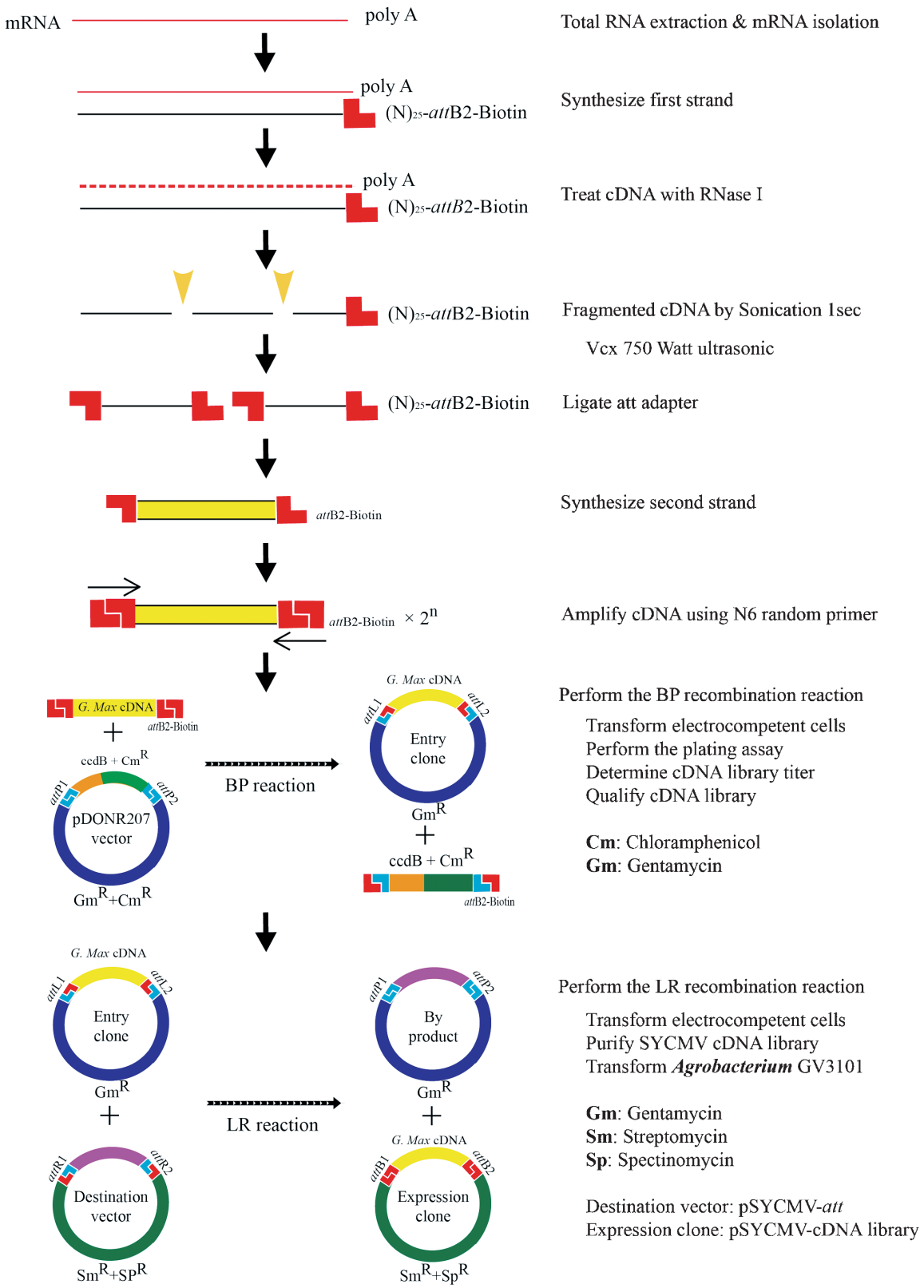

Fig. 1. Experiment flowchart. Generation of an SYCMV cDNA library follows this process. Total RNA was extracted from Soybean (*Glycine max*) and mRNA isolation was performed with FastTrack MAG mRNA isolation Kit (Invitrogen). First strand cDNA was synthesized using isolated mRNA and random primer $(N)_{25}$–*att*B2–Biotin and then treated with RNase I to digest RNA. The resulting first strand cDNA was fragmented by sonication for 1 second with a Vcx 750 Watt ultrasonic probe. The ends of the resulting small fragments (250–500 nt) were ligated with annealed ATT1 adapter oligomers using T4 DNA ligase and then the second strands synthesized by extension using Taq™ DNA polymerase. The double–strand cDNA fragments were amplified using 5'*att*B1 and Biotin–*att*B2–$(N)_6$ primers, and used to generate a cDNA library of fragments suitable for RNAi. The SYCMV vector was modified with an MCS in the 3' non–coding region for subsequent Gateway insertion of soybean cDNA RNAi constructs. The soybean cDNA fragments were transferred to the Gateway–modified pSYCMV–*att* vector via the BP and LR recombination in order to generate RNAi in infected plants. The resulting pSYCMV–*att* cDNA library has potential for high–throughput gene function identification in soybean.

BP RECOMBINATION

BP recombination was performed with 4.8 μg of the *att*B–flanked amplified cDNA fragments and 4.5 μg of pDONR™ 207 vector (Invitrogen). The product of recombination reactions (BP reactions) was used to transform One shot® Top10 electrocompetent cells E.coli (Invitrogen) by electroporator using settings of 1.8 kV, 200 Ω, 25 uF in a final volume of 50 μl, and then brought to 1 ml with S.O.C. media and incubated at 37°C for 1 hour. Then 100 μl of the cDNA library was spread on LB plates containing 1 μg/ml gentamycin. Plates were incubated overnight at 37°C, and then the cDNA library titer determined by counting colonies. We selected twenty random colonies of *E. coli* for to determine redundancy and insert size by PCR analysis on agarose gel using purified plasmid as template. The plasmids prepared from these randomly–selected colonies were also submitted for sequencing to evaluate library diversity (Table 2). The remainder of the cDNA library (900 μl) was divided into 300 μl aliquots, to which was added an equal volume of sterile 40% glycerol prior to storage at – 80°C.

A MULTIPLE CLONING SITE AND GATEWAY CLONING ADAPTER WERE INSERTED IN THE SYCMV 3' NON–CODING REGION

A *BsrGI* restriction site was previously introduced into the 3' non–coding region of the SYCMV infectious clone to generate VIGS vector pSYCMV–full–*BsrGI* (Nam *et al.*, 2012; and unpublished). A multiple cloning site (MCS) including *Stu*I, *Spe*I, *Swa*I, and *Apa*I sites was designed with overlapping oligomers (Table 1; synthe-

Table 2. EST Data of *G. max* cDNA library; analysis of 20 BP reaction clones for redundancy and insert sequence

No.	Acc No. (Length)	EST data	Range (Identities)
1	GD346518 (66bp)	454PCEP0056674 Scarlet Runner Bean globular–stage embryo–proper regions, *Phaseolus coccineus* cDNA	1–65 (91%)
2	DB956718 (664bp)	DB956718 full–length enriched soybean cDNA library, mixture of seedling, young plant, adult plant , *Glycine max* cDNA	2–410 (99%)
3	EH038544 (862bp)	GMSeed1261 Soybean endosperm tissue in developing seeds, *Glycine max* cDNA	653–741 (98%)
4	FK007193 (775bp)	GLMDW20TR JCVI–SOY2, *Glycine max* cDNA	98–306 (100%)
5	EV277683 (603bp)	GLMD702TF JCVI–SOY2, *Glycine max* cDNA	4–281 (100%)
6	DB985877 (536bp)	DB985877 full–length enriched soybean cDNA library, mixture of seedling, young plant, adult plant, *Glycine max* cDNA	81–456 (99%)
7	AB863401 (922bp)	AB863401 Lotus tenuis leaf Lotus tenuis cDNA.	12–91 (99%)
8	HO020324 (577bp)	ocpsga0_0152_A11.ab1 Soybean immature seed full–length–enriched cDNA library, *Glycine max* cDNA.	220–397 (100%)
9	HO794097 (1,178bp)	vsu–ars_019130_LI15contig_289 VSU–ARS–L15 *Phaseolus acutifolius* cDNA.	360–1010 (99%)
10	BW672753 (477bp)	BW672753 GMFL01 *Glycine max* cDNA.	1–328 (99%)
11	EV278297 (748bp)	GLNA517TF JCVI–SOY3 *Glycine max* cDNA.	16–250 (89%)
12	DB972543 (624bp)	DB972543 full–length enriched soybean cDNA library, mixture of seedling, young plant, adult plant *Glycine max* cDNA	2–195 (97%)
13	FK020246 (857bp)	GLNDK07TF JCVI–SOY3, *Glycine max* cDNA	336–855 (99%)
14	GR859176 (783bp)	CCCC22589.b1 CCCC Glycine max callus grown in dark condition, *Glycine max* cDNA	1–123 (100%)
15	HO030346 (559bp)	ocpsga0_0280_H01.ab1 Soybean immature seed full–length–enriched cDNA library *Glycine max* cDNA	145–406 (100%)
16	DB965949 (591bp)	DB965949 full–length enriched soybean cDNA library, mixture of seedling, young plant, adult plant, *Glycine max* cDNA	36–226 (97%)
17	AB863401 (922bp)	AB863401 Lotus tenuis leaf Lotus tenuis cDNA.	12–91 (99%)
18		Unknown	
19		Unknown	
20		Unknown	

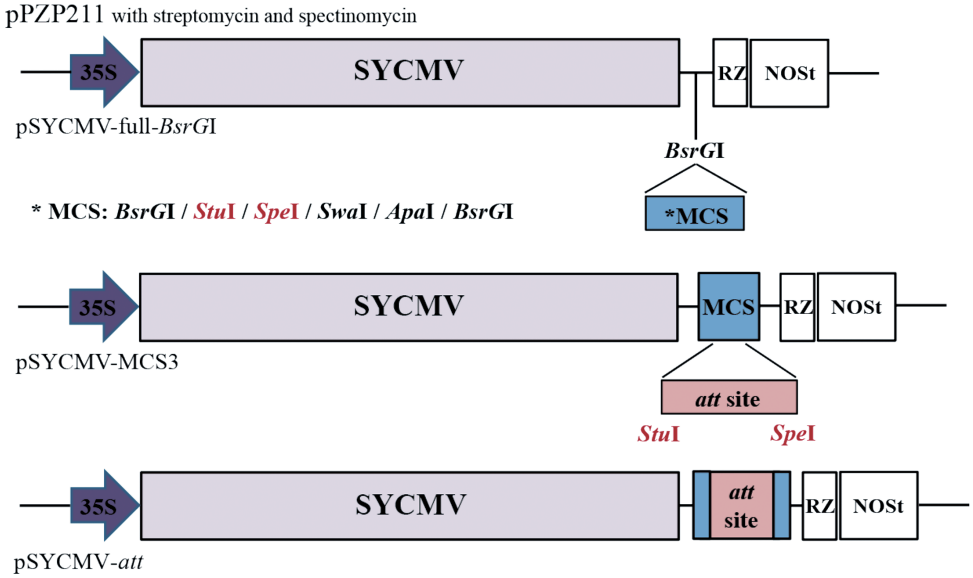

Fig. 2. Generation of a Gateway cloning site in the pSYCMV 3' non–cording region. SYCMV *BsrGI* site in non–coding region was supplied by Nam *et al*. (2012, and unpublished) In order to create a multiple cloning site we designed self–complementary oligomers (Table 1), which were annealed and inserted to *BsrGI* cut SYCMV vector, creating pSYCMV–MCS3. The Gateway *att* site was amplified with primers containing *Stu*I and *Spe*I restriction sites, and the PCR product was inserted to *Stu*I and *Spe*I cut pSYCMV–MCS3, generating pSYCMV–*att*.

sized by Macrogen) generating *BsrGI* cohesive ends; 10 pmol of each of these oligomers were mixed and denatured at 95°C for 5 minutes in a thermal cycler, followed by gradual cooling to room temperature. The annealed oligomers were then ligated to pSYCMV–full–*BsrGI* digested with *BsrGI*, creating pSYCMV–MCS3. A Gateway cloning adapter was amplified from pDONR207 vector by PCR using primers adding *Stu*I and *Spe*I respectively, and ligated into pSYCMV–MCS3 after digestion of both vector and PCR product with *Stu*I and *Spe*I, generating the pSYCMV–*att* VIGS vector (Figure 2) with the Gateway system to be utilized for soybean gene function analysis.

LR RECOMBINATION

Harvested BP library DNA (1µg) was used directly for LR recombination with 1 μg of pSYCMV–*att*, transformed to electrocompetent *E. coli*, and plated on SM+SP media (Figure 1). All of the resultant colonies were harvested by flushing from the plate in buffer and the purified SYCMV–*att* cDNA library (1.415 μg) was transformed into Agrobacterium GV3101.

AGROINOCULATION OF *Glycine max* WITH SYCMV–*att* CONTAINING RANDOM cDNA INSERTS

Individual Agrobacterium colonies from the SYCMV–*att* cDNA library were streaked onto RIF+SM+SP agar plates and incubated at 30°C for 2 days. Agrobacterium cells were harvested from the plate with inoculation loops, resuspended in infiltration buffer (10 mM MES, (pH 5.6), 10 mM $MgCl_2$ and 150 mM acetosyringone) at OD_{600} = 0.6. Three 10–day old *G. max* seedlings in a single pot were agroinfiltrated with each selected SYCMV–Att cDNA construct on both cotyledons and primary leaf using a total of 5 ml Agrobacterium suspension.

RESULTS AND DISCUSSION

CREATION OF A RANDOM *GLYCINE MAX* CDNA LIBRARY IN *E. COLI* USING CDNA SONICATION, UNBIASED AMPLIFICATION, AND GATEWAY CLONING INTO THE SYCMV–*att* VIGS VECTOR

A random, unbiased cDNA library was generated from soybean mRNA by cDNA sonication to yield fragments predominantly in the range of 250–500 nt, followed by amplification to add Gateway adapters. These fragments were transferred to the Gateway donor plasmid pDONR207 by the BP reaction, and 20 random colonies from the resulting cDNA library analyzed for insert size. The sequences of these randomly–selected colonies revealed that 18 represented unique genes (of which three were notably not identified by the Blast search, and thus appear to represent previously unknown genes); the remaining two represent different regions of a sequence most closely related to AB863401 (Table 2), a *Lotus tenuis* rRNA transcript. The initial BP reaction library was therefore shown to have a low degree of redundancy.

A *GLYCINE MAX* CDNA LIBRARY WAS SUCCESSFULLY ESTABLISHED IN THE GATEWAY–MODIFIED SYCMV VECTOR in AGROBACTERIUM

The BP cDNA library was next purified and transferred to the SYCMV–*att* vector by the Gateway LR reac-

tion, and transformed initially into *E. coli*, from which plasmid DNA was purified and used to transform Agrobacterium GV3101. From 1.415 μg of LR reaction SYCMV–*att* library purified from *E. coli*, 632 colonies of Agrobacterium were recovered. From these colonies, approximately 320 were selected for amplification by colony PCR and PCR sequencing; from these amplified fragments, high quality sequence data was obtained from 199 colonies (Table 3). In contrast to the results from the BP reactions, in which a low degree of redundancy was observed, only 21 of 119 Agrobacterium clones (17.7%) from which high quality sequence was obtained were derived from unique cDNAs, whereas 98 other clones were derived from a total of 15 additional cDNAs (a total of 36 distinct cDNAs; Table 3). There were a total of 13 clones which had identity to BI470420, 12 with identity to BW664164, 11 to HO010297, and 10 to FK009800 (Table 3). It is probable that the degree of redundancy could be reduced significantly if the LR reaction were to be transformed directly into Agrobacterium instead of initially into *E. coli*, but the transformation efficiency of Agrobacterium is significantly lower than that of *E. coli*.

Table 3. Colony PCR sequencing and BLAST search results of SYCMV cDNA library

No.	Acc No. (Length)	EST data	Range (Identities)
7	CD410772.1 (452bp)	Gm_ck3907 Soybean induced by Salicylic Acid Glycine max cDNA 3–, mRNA sequence	96–385(99%)
9	HO026154.1 (543 bp)	ocpsga0_0226_H05.ab1 Soybean immature seed full–length–enriched cDNA library Glycine max cDNA, mRNA sequence	198–352(97%)
48	BI941834.1 (466 bp)	sd38f02.y1 Gm–c1016 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm–c1016–2068 5– similar to TR:O14597 O14597 NON–FUNCTIONAL FOLATE BINDING PROTEIN. ;, mRNA sequence	314–445(90%)
50	FK009800.1 (718 bp)	GLQA601TF JCVI–SOY2 Glycine max cDNA 5–, mRNA sequence	45–559(98%)
51	FK631391.1 (91 bp)	454GmaGlobSeed366023 Soybean Seeds Containing Globular–Stage Embryos Glycine max cDNA, mRNA sequence	11–88(94%)
52	BW664164.1 (292 bp)	BW664164 GMFL01 Glycine max cDNA clone GMFL01–22–M23 5–, mRNA sequence	1–165(98%)
53	EV272763.1 (796 bp)	GLMBN76TF JCVI–SOY2 Glycine max cDNA 5–, mRNA sequence	73–382(97%)
54	BW664164.1 (292 bp)	BW664164 GMFL01 Glycine max cDNA clone GMFL01–22–M23 5–, mRNA sequence	1–215(98%)
57	HX900774.1 (836 bp)	HX900774 Vicia Faba cultivar Komasakae ethiolated seedlings Vicia Faba cDNA, mRNA sequence	71–732(99%)
59	BI470420.1 (538 bp)	sah91g01.y1 Gm–c1050 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm–c1050–3386 5– similar to SW:Y53L_SYNY3 P72583 YCF53–LIKE PROTEIN. ;, mRNA sequence	17–127(99%)
63	BI470420.1 (538 bp)	sah91g01.y1 Gm–c1050 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm–c1050–3386 5– similar to SW:Y53L_SYNY3 P72583 YCF53–LIKE PROTEIN. ;, mRNA sequence	16–127(99%)
64	FK631391.1 (91 bp)	454GmaGlobSeed366023 Soybean Seeds Containing Globular–Stage Embryos Glycine max cDNA, mRNA sequence.	12–88(94%)
66	BE661686.1 (636 bp)	0–D2 GmaxSC Glycine max cDNA, mRNA sequence	184–371(97%)
67	HO761426.1 (814 bp)	B100430573–3–5–H6–M13_B12.ab1 SSH cDNA library induced by salt from soybean Glycine max cDNA, mRNA sequence	44–260(99%)
68	CK606111.1 (389 bp)	gmrhRww6–08_C12_T7 Soybean root hair subtracted cDNA library gmrhRww6 Glycine max cDNA, mRNA sequence	50–361(97%)
69	FK009800.1 (718 bp)	GLQA601TF JCVI–SOY2 Glycine max cDNA 5–, mRNA sequence	45–339(94%)
73	BW664164.1 (292 bp)	BW664164 GMFL01 Glycine max cDNA clone GMFL01–22–M23 5–, mRNA sequence	1–164(94%)
74	HO010297.1 (553 bp)	ocpsga0_0024_C02.ab1 Soybean immature seed full–length–enriched cDNA library Glycine max cDNA, mRNA sequence	223–333(100%)
75	BI470420.1 (538 bp)	sah91g01.y1 Gm–c1050 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm–c1050–3386 5– similar to SW:Y53L_SYNY3 P72583 YCF53–LIKE PROTEIN. ;, mRNA sequence	16–127(99%)
76	BI470420.1 (538 bp)	sah91g01.y1 Gm–c1050 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm–c1050–3386 5– similar to SW:Y53L_SYNY3 P72583 YCF53–LIKE PROTEIN. ;, mRNA sequence	16–127(99%)

No.	Acc No. (Length)	EST data	Range (Identities)
77	BF716250.1 (589 bp)	saa16g10.y1 Gm–c1058 Glycine soja cDNA clone GENOME SYSTEMS CLONE ID: Gm–c1058–1484 5– similar to TR:Q9SML3 Q9SML3 CYTOCHROME P450 MONOOXYGENASE ;, mRNA sequence	16–497(99%)
78	CD410772.1 (452 bp)	Gm_ck3907 Soybean induced by Salicylic Acid Glycine max cDNA 3–, mRNA sequence	96–385(98%)
80	EV272763.1 (796 bp)	GLMBN76TF JCVI–SOY2 Glycine max cDNA 5', mRNA sequence.	73–382(99%)
82	BW664164.1 (292 bp)	BW664164 GMFL01 Glycine max cDNA clone GMFL01–22–M23 5–, mRNA sequence	1–173(99%)
83	HO011223.1 (587 bp)	ocpsga0_0035_F10.ab1 Soybean immature seed full–length–enriched cDNA library Glycine max cDNA, mRNA sequence	281–558(95%)
84	HO010297.1 (553 bp)	ocpsga0_0024_C02.ab1 Soybean immature seed full–length–enriched cDNA library Glycine max cDNA, mRNA sequence	217–333(99%)
85	HO010297.1 (553 bp)	ocpsga0_0024_C02.ab1 Soybean immature seed full–length–enriched cDNA library Glycine max cDNA, mRNA sequence	217–333(99%)
86	CD410772.1 (452 bp)	Gm_ck3907 Soybean induced by Salicylic Acid Glycine max cDNA 3–, mRNA sequence	96–385(99%)
87	HO010297.1 (553 bp)	ocpsga0_0024_C02.ab1 Soybean immature seed full–length–enriched cDNA library Glycine max cDNA, mRNA sequence	223–333(100%)
88	HO010297.1 (553 bp)	ocpsga0_0024_C02.ab1 Soybean immature seed full–length–enriched cDNA library Glycine max cDNA, mRNA sequence	223–333(100%)
89	BW664164.1 (292 bp)	BW664164 GMFL01 Glycine max cDNA clone GMFL01–22–M23 5–, mRNA sequence	1–215(98%)
90	HO792335.1 (868 bp)	vsu–ars_017368_LI10Contig_28919 VSU–ARS–L10 Phaseolus acutifolius cDNA, mRNA sequence	247–365(98%)
91	HO776593.1 (650 bp)	vsu–ars_001626_LI10B00564 VSU–ARS–L10 Phaseolus acutifolius cDNA, mRNA sequence	204–574(97%)
92	HX900774.1 (836 bp)	HX900774 Vicia Faba cultivar Komasakae ethiolated seedlings Vicia Faba cDNA, mRNA sequence	366–732(96%)
95	BI470420.1 (538 bp)	sah91g01.y1 Gm–c1050 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm–c1050–3386 5– similar to SW:Y53L_SYNY3 P72583 YCF53–LIKE PROTEIN. ;, mRNA sequence	16–127(99%)
97	FK631391.1 (91 bp)	454GmaGlobSeed366023 Soybean Seeds Containing Globular–Stage Embryos Glycine max cDNA, mRNA sequence	11–88(95%)
99	CK606111.1 (389 bp)	gmrhRww6–08_C12_T7 Soybean root hair subtracted cDNA library gmrhRww6 Glycine max cDNA, mRNA sequence.	50–361(99%)
100	EV272763.1 (796 bp)	GLMBN76TF JCVI–SOY2 Glycine max cDNA 5', mRNA sequence.	73–382(96%)
101	HO010297.1 (553 bp)	ocpsga0_0024_C02.ab1 Soybean immature seed full–length–enriched cDNA library Glycine max cDNA, mRNA sequence	223–333(100%)
107	BM526195.1 (424 bp)	sal38b08.y1 Gm–c1059 Glycine soja cDNA clone SOYBEAN CLONE ID: Gm–c1059–4311 5' similar to TR:Q05929 Q05929 EXTRACELLULAR DERMAL GLYCOPROTEIN PRECURSOR ;, mRNA sequence.	200–328(93%)
109	HO792335.1 (868 bp)	vsu–ars_017368_LI10Contig_28919 VSU–ARS–L10 Phaseolus acutifolius cDNA, mRNA sequence	247–365(98%)
110	CD410772.1 (452 bp)	Gm_ck3907 Soybean induced by Salicylic Acid Glycine max cDNA 3–, mRNA sequence	96–385(99%)
113	CD410772.1 (452 bp)	Gm_ck3907 Soybean induced by Salicylic Acid Glycine max cDNA 3–, mRNA sequence	96–352(97%)
116	HO010297.1 (553 bp)	ocpsga0_0024_C02.ab1 Soybean immature seed full–length–enriched cDNA library Glycine max cDNA, mRNA sequence	217–333(98%)
117	BI945945.1 (434 bp)	st52g11.y1 Gm–c1053 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm–c1053–334 5' similar to TR:Q40335 Q40335 ALFALFA BIMODULAR PROTEIN ;, mRNA sequence.	34–212(97%)
119	BI470420.1 (538 bp)	sah91g01.y1 Gm–c1050 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm–c1050–3386 5– similar to SW:Y53L_SYNY3 P72583 YCF53–LIKE PROTEIN. ;, mRNA sequence	17–127(99%)
123	HX900342.1 (866 bp)	HX900342 Vicia Faba cultivar Komasakae ethiolated seedlings Vicia Faba cDNA, mRNA sequence.	395–619(98%)
125	HO792335.1 (868 bp)	vsu–ars_017368_LI10Contig_28919 VSU–ARS–L10 Phaseolus acutifolius cDNA, mRNA sequence	247–365(98%)

No.	Acc No. (Length)	EST data	Range (Identities)
126	BI470420.1 (538 bp)	sah91g01.y1 Gm–c1050 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm–c1050–3386 5– similar to SW:Y53L_SYNY3 P72583 YCF53–LIKE PROTEIN. ;, mRNA sequence	16–127(99%)
28	FK009800.1 (718 bp)	GLQA601TF JCVI–SOY2 Glycine max cDNA 5', mRNA sequence.	47–560(98%)
129	FK009800.1 (718 bp)	GLQA601TF JCVI–SOY2 Glycine max cDNA 5–, mRNA sequence	45–560(93%)
133	CA785173.1 (742 bp)	sau25f02.y1 Gm–c1062 Glycine max cDNA clone SOYBEAN CLONE ID: Gm–c1062–9459 5' similar to TR:Q9SML3 Q9SML3 CYTOCHROME P450 MONOOXYGENASE ;, mRNA sequence.	77–408(98%)
134	HO804963.1 (1,019 bp)	vsu–ars_029996_LI15contig_27507 VSU–ARS–L15 Phaseolus acutifolius cDNA, mRNA sequence.	116–446(95%)
135	BE661686.1 (636 bp)	0–D2 GmaxSC Glycine max cDNA, mRNA sequence.	184–371(97%)
138	FK009800.1 (718 bp)	GLQA601TF JCVI–SOY2 Glycine max cDNA 5–, mRNA sequence	45–376(89%)
142	BI972805.1 (423 bp)	sai83c04.y1 Gm–c1065 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm–c1065–7423 5', mRNA sequence.	107–372(97%)
143	CK606111.1 (389 bp)	gmrhRww6–08_C12_T7 Soybean root hair subtracted cDNA library gmrhRww6 Glycine max cDNA, mRNA sequence.	50–361(99%)
144	CD410772.1 (452 bp)	Gm_ck3907 Soybean induced by Salicylic Acid Glycine max cDNA 3–, mRNA sequence	88–385(92%)
145	HO026154.1 (543 bp)	ocpsga0_0226_H05.ab1 Soybean immature seed full–length–enriched cDNA library Glycine max cDNA, mRNA sequence	198–388(96%)
146	HO792335.1 (868 bp)	vsu–ars_017368_LI10Contig_28919 VSU–ARS–L10 Phaseolus acutifolius cDNA, mRNA sequence	247–365(98%)
147	BW664164.1 (292 bp)	BW664164 GMFL01 Glycine max cDNA clone GMFL01–22–M23 5–, mRNA sequence	1–215(98%)
149	FK009800.1 (718 bp)	GLQA601TF JCVI–SOY2 Glycine max cDNA 5', mRNA sequence.	45–335(90%)
151	EV272763.1 (796 bp)	GLMBN76TF JCVI–SOY2 Glycine max cDNA 5', mRNA sequence.	73–379(92%)
152	BI470420.1 (538 bp)	sah91g01.y1 Gm–c1050 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm–c1050–3386 5– similar to SW:Y53L_SYNY3 P72583 YCF53–LIKE PROTEIN. ;, mRNA sequence	16–127(99%)
153	FK005849.1 (587 bp)	GLMDO74TR JCVI–SOY2 Glycine max cDNA 5', mRNA sequence.	61–220(95%)
155	BI470420.1 (538 bp)	sah91g01.y1 Gm–c1050 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm–c1050–3386 5– similar to SW:Y53L_SYNY3 P72583 YCF53–LIKE PROTEIN. ;, mRNA sequence	16–127(99%)
156	BW664164.1 (292 bp)	BW664164 GMFL01 Glycine max cDNA clone GMFL01–22–M23 5–, mRNA sequence	1–215(99%)
160	EV272763.1 (796 bp)	GLMBN76TF JCVI–SOY2 Glycine max cDNA 5', mRNA sequence.	73–382(92%)
161	DB968002.1 (405 bp)	DB968002 full–length enriched soybean cDNA library, mixture of seedling, young plant, adult plant Glycine max cDNA clone GMFL02–36–C01 5', mRNA sequence.	147–400(98%)
162	EV272763.1 (796 bp)	GLMBN76TF JCVI–SOY2 Glycine max cDNA 5', mRNA sequence.	73–378(92%)
164	FK005849.1 (587 bp)	GLMDO74TR JCVI–SOY2 Glycine max cDNA 5', mRNA sequence.	7–213(99%)
165	AW132685.1 (310 bp)	se08g02.y1 Gm–c1013 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm–c1013–2955 5' similar to TR:O64835 O64835 F27L4.14 PROTEIN. ;, mRNA sequence.	173–307(99%)
167	CK606111.1 (389 bp)	gmrhRww6–08_C12_T7 Soybean root hair subtracted cDNA library gmrhRww6 Glycine max cDNA, mRNA sequence.	50–361(99%)
168	HO043480.1 (533 bp)	ocpsga0_0480_E10.ab1 Soybean immature seed full–length–enriched cDNA library Glycine max cDNA, mRNA sequence.	86–348(94%)
169	BI972805.1 (423 bp)	sai83c04.y1 Gm–c1065 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm–c1065–7423 5', mRNA sequence.	92–372(99%)
176	FK631391.1 (91 bp)	454GmaGlobSeed366023 Soybean Seeds Containing Globular–Stage Embryos Glycine max cDNA, mRNA sequence	11–88(94%)

No.	Acc No. (Length)	EST data	Range (Identities)
178	HO010297.1 (553 bp)	ocpsga0_0024_C02.ab1 Soybean immature seed full–length–enriched cDNA library Glycine max cDNA, mRNA sequence	223–333(100%)
179	HO792335.1 (868 bp)	vsu–ars_017368_LI10Contig_28919 VSU–ARS–L10 Phaseolus acutifolius cDNA, mRNA sequence	247–365(98%)
181	FK009800.1 (718 bp)	GLQA601TF JCVI–SOY2 Glycine max cDNA 5–, mRNA sequence	45–356(93%)
183	CK605660.1 (383 bp)	gmrhRww6–02_E11_T7 Soybean root hair subtracted cDNA library gmrhRww6 Glycine max cDNA, mRNA sequence.	108–334(97%)
185	HX900774.1 (836 bp)	HX900774 Vicia Faba cultivar Komasakae ethiolated seedlings Vicia Faba cDNA, mRNA sequence.	392–732(91%)
188	HO026203.1 (506 bp)	ocpsga0_0227_E03.ab1 Soybean immature seed full–length–enriched cDNA library Glycine max cDNA, mRNA sequence.	75–255(97%)
189	FK009800.1 (718 bp)	GLQA601TF JCVI–SOY2 Glycine max cDNA 5', mRNA sequence.	45–379(96%)
191	BI972805.1 (423 bp)	sai83c04.y1 Gm–c1065 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm–c1065–7423 5', mRNA sequence.	162–372(99%)
192	HX900774.1 (836 bp)	HX900774 Vicia Faba cultivar Komasakae ethiolated seedlings Vicia Faba cDNA, mRNA sequence	71–717(98%)
194	HO807365.1 (322 bp)	vsu–ars_032398_LI15contig_30213 VSU–ARS–L15 Phaseolus acutifolius cDNA, mRNA sequence.	3–313(94%)
195	FK005849.1 (587 bp)	GLMDO74TR JCVI–SOY2 Glycine max cDNA 5', mRNA sequence.	7–220(99%)
196	BI972805.1 (423 bp)	sai83c04.y1 Gm–c1065 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm–c1065–7423 5', mRNA sequence.	154–372(99%)
201	HO792335.1 (868 bp)	vsu–ars_017368_LI10Contig_28919 VSU–ARS–L10 Phaseolus acutifolius cDNA, mRNA sequence	247–365(98%)
205	BQ080845.1 (552 bp)	san11a03.y1 Gm–c1084 Glycine max cDNA clone SOYBEAN CLONE ID: Gm–c1084–3462 5' similar to TR:O81397 O81397 CRP1. ;, mRNA sequence.	217–333(98%)
208	BW664164.1 (292 bp)	BW664164 GMFL01 Glycine max cDNA clone GMFL01–22–M23 5', mRNA sequence.	1–66(95%)
203	HO010297.1 (553 bp)	ocpsga0_0024_C02.ab1 Soybean immature seed full–length–enriched cDNA library Glycine max cDNA, mRNA sequence	1–202(98%)
215	BW664164.1 (292 bp)	BW664164 GMFL01 Glycine max cDNA clone GMFL01–22–M23 5–, mRNA sequence	1–204(99%)
217	HX914114.1 (842 bp)	HX914144 Vicia Faba cultivar Komasakae roots Vicia Faba cDNA, mRNA sequence.	239–439(98%)
219	FK005849.1 (587 bp)	GLMDO74TR JCVI–SOY2 Glycine max cDNA 5', mRNA sequence.	7–221(94%)
221	BI470420. (538 bp)	sah91g01.y1 Gm–c1050 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm–c1050–3386 5' similar to SW:Y53L_SYNY3 P72583 YCF53–LIKE PROTEIN. ;, mRNA sequence.	16–127(99%)
229	HO010297.1 (553 bp)	ocpsga0_0024_C02.ab1 Soybean immature seed full–length–enriched cDNA library Glycine max cDNA, mRNA sequence	217–333(98%)
230	FK631391.1 (91 bp)	454GmaGlobSeed366023 Soybean Seeds Containing Globular–Stage Embryos Glycine max cDNA, mRNA sequence	11–88(94%)
231	DB964958.1 (528 bp)	DB964958 full–length enriched soybean cDNA library, mixture of seedling, young plant, adult plant Glycine max cDNA clone GMFL02–27–H08 5', mRNA sequence.	128–372(99%)
232	HX900774.1 (836 bp)	HX900774 Vicia Faba cultivar Komasakae ethiolated seedlings Vicia Faba cDNA, mRNA sequence	71–717(98%)
237	BI470420.1 (538 bp)	sah91g01.y1 Gm–c1050 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm–c1050–3386 5' similar to SW:Y53L_SYNY3 P72583 YCF53–LIKE PROTEIN. ;, mRNA sequence.	16–127(99%)
251	EV272763.1 (796 bp)	GLMBN76TF JCVI–SOY2 Glycine max cDNA 5–, mRNA sequence	73–382(97%)
252	BU084341.1 (571 bp)	sar18c07.y1 Gm–c1049 Glycine max cDNA clone SOYBEAN CLONE ID: Gm–c1049–7021 5' similar to TR:Q9SU29 Q9SU29 POLYUBIQUITIN–LIKE PROTEIN. ;, mRNA sequence.	3–291(98%)
253	BI470420.1 (538 bp)	sah91g01.y1 Gm–c1050 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm–c1050–3386 5' similar to SW:Y53L_SYNY3 P72583 YCF53–LIKE PROTEIN. ;, mRNA sequence.	16–127(99%)

No.	Acc No. (Length)	EST data	Range (Identities)
254	AW133096.1 (549 bp)	se14c08.y1 Gm–c1013 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm–c1013–3495 5' similar to SW:Y53L_SYNY3 P72583 YCF53–LIKE PROTEIN. ;, mRNA sequence.	4–116(99%)
257	EV272763.1 (796 bp)	GLMBN76TF JCVI–SOY2 Glycine max cDNA 5–, mRNA sequence	73–382(97%)
259	FK009800.1 (718 bp)	GLQA601TF JCVI–SOY2 Glycine max cDNA 5–, mRNA sequence	45–560(98%)
262	GR851326.1 (727 bp)	CCCC18087.g1 CCCC Glycine max callus grown in dark condition Glycine max cDNA clone CCCC18087 3', mRNA sequence.	296–555(93%)
270	BI470420.1 (538 bp)	sah91g01.y1 Gm–c1050 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm–c1050–3386 5' similar to SW:Y53L_SYNY3 P72583 YCF53–LIKE PROTEIN. ;, mRNA sequence.	17–127(99%)
272	HO010297.1 (553 bp)	ocpsga0_0024_C02.ab1 Soybean immature seed full–length–enriched cDNA library Glycine max cDNA, mRNA sequence.	217–333(97%)
275	BW664164.1 (292 bp)	BW664164 GMFL01 Glycine max cDNA clone GMFL01–22–M23 5–, mRNA sequence	1–215(96%)
277	BW664164.1 (292 bp)	BW664164 GMFL01 Glycine max cDNA clone GMFL01–22–M23 5–, mRNA sequence	1–204(96%)
279	BI972805.1 (423 bp)	sai83c04.y1 Gm–c1065 Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm–c1065–7423 5', mRNA sequence.	96–372(99%)
281	FK009800.1 (718 bp)	GLQA601TF JCVI–SOY2 Glycine max cDNA 5–, mRNA sequence	48–287(92%)
282	FK005849.1 (587 bp)	GLMDO74TR JCVI–SOY2 Glycine max cDNA 5', mRNA sequence	7–220(99%)
284	BW664164.1 (292 bp)	BW664164 GMFL01 Glycine max cDNA clone GMFL01–22–M23 5–, mRNA sequence	1–203(99%)
285	HO026203.1 (506 bp)	ocpsga0_0227_E03.ab1 Soybean immature seed full–length–enriched cDNA library Glycine max cDNA, mRNA sequence.	83–255(91%)
287	HO014584.4 (517 bp)	ocpsga0_0077_D02.ab1 Soybean immature seed full–length–enriched cDNA library Glycine max cDNA, mRNA sequence.	7–180(99%)
288	FK631391.1 (91 bp)	454GmaGlobSeed366023 Soybean Seeds Containing Globular–Stage Embryos Glycine max cDNA, mRNA sequence.	11–88(94%)

INOCULATION OF *GLYCINE MAX* WITH SYCMV–*ATT* EXPRESSING RANDOMLY–SELECTED RNAi CONSTRUCTS

About 320 randomly–selected colonies containing SYCMV–*Att* expressing RNAi were agro–inoculated to both cotyledons and first true leaves of three separate *Glycine max* seedlings per construct. At 45 dpi we selected those plants producing distinctive visible phenotypes among SYCMV infected soybeans (Figure 3). Soybean infected with wild type SYCMV and SYCMV–*att* controls showed slight stunting, with mottled yellow green leaves as compared with healthy soybean. SYCMV VIGS 15 and 189 plants were more dwarfed, and with smaller leaves owing to retarded growth. Plants infected with SYCMV VIGS 102 and 113 had fewer leaves which were also lighter green than the SYCMV and SYCMV–*att* controls, and failed to develop flowers (Figure 3). Analysis of the inserted RNAi sequences of those clones yielding visible phenotypes, by reverse transcription PCR (using pSYCMV *att* B1 and B2 primers; Table 1) from RNA extracted from SYCMV–VIGS infected soy-

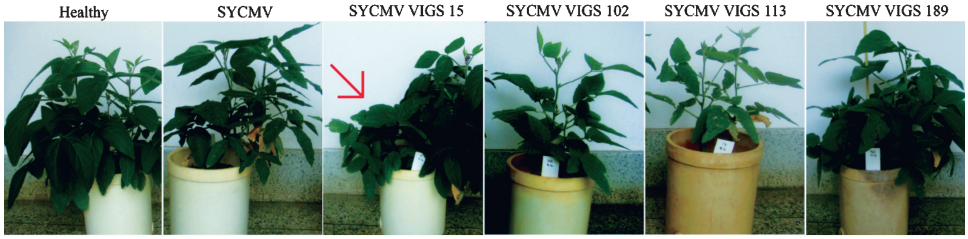

Fig. 3. Soybeans were selected among SYCMV infected plants producing distinctively visible phenotype. SYCMV has typical symptoms of yellow–green mottle and mosaic. Soybeans infected with SYCMV VIGS 15 and SYCMV VIGS 189 showed stunting as compared with healthy plants. Soybeans infected with SYCMV VIGS 102 and SYCMV VIGS 113 produced fewer leaves and floral initiation was absent.

Table 4. BLAST search data of SYCMV VIGS constructs containing *G. max* genes which showed distinct visible phenotypes

No.	Acc No.	EST Data
15	BU761402.1[a]	sas71e02.y1 Gm–c1036 *Glycine max* cDNA clone SOYBEAN CLONE ID: Gm–c1036–9795 5–similar to TR:O23345 O23345 HYDROXYPROLINE–RICH GLYCOPROTEIN HOMOLOG., mRNA sequence
102	CD412470.1[b]	Gm_ck43584 Soybean induced by Salicylic *Acid Glycine max* cDNA 3', mRNA sequence.
113	XM_003532354.2[c]	*Glycine max* acyl carrier protein 1, chloroplastic–like (LOC100806925), mRNA
189	XM_003542836.2[d]	*Glycine max* basic 7S globulin–like (LOC100807749), mRNA

[a] BLAST analysis of this EST revealed 98% identity to a soybean genomic clone identified as FRIGIDA–like protein 4a–like (XM_003556002)
[b] BLAST analysis of this EST revealed 99% identity to a soybean genomic clone (XM_003546474) of unknown function.
[c] This genomic clone has 100% identity to the EST (CD410772) identified for the same clone in Table 3.
[d] This genomic clone has 99% identity to the EST (FK009800) identified for the same clone in Table 3.

beans and BLAST search against the data base identified two out of four genes (Table 4) that were not identified by sequencing of colony PCR amplicons obtained directly from Agrobacterium (Table 3), as they were among those for which sequencing failed to yield high quality data from colony PCR.

The phenotype observed with SYCMV VIGS 102 and 113, failure to develop flowers, would clearly be of significant economic importance in any crop, and especially in soybean where the seed is the primary yield. Clone 102 was identified as a gene of unknown function induced by salicylic acid (EST CD412470, genomic sequence XM_003546474) having an N–terminal–proximal transmembrane domain but cytoplasmic localization (PredictProtein server; Goldberg *et al*., 2012; Rost *et al*., 1996). Clone 113 was identified as a chloroplast–localized (PredictProtein server; Goldberg *et al*., 2012) acyl carrier protein (XM003532354) (Table 4). No localization was predicted for clone 189, but an N–terminal transmembrane domain was identified, and a potential role in proteolysis (PredictProtein server; Goldberg *et al*., 2012; Hamp *et al*., 2013; Rost *et al*., 1996). Interestingly, clone 15 was initially identified as having identity to an EST having a hydroxyproline rich glycoprotein homolog (BU761402, Table 4), but BLAST analysis with this EST sequence showed 98% identity to a soybean genomic clone identified as FRIGIDA–like protein 4a–like (XM_003556002), which is predicted to have a nuclear localization (PredictProtein server; Goldberg *et al*., 2012); FRIGIDA itself has been shown to be involved in variation of flowering time in Arabidopsis (Johanson *et al*., 2000), so it is possible that the soybean represented by clone 15 is also involved in flowering time. The roles of these genes in floral development and other functions will be investigated further in on–going work.

At least 63,000 ESTs have previously been identified from *Glycine max* (Hisano *et al*., 2007), and functional annotation of all of these ESTs will take considerable time in the absence of identity to orthologs from other species. Reverse genetics using a VIGS vector offers an alternative means of identifying function of an unknown gene (Schofield and Nelson, 2009). However, the approach to identify individual genes requires specific amplification and cloning of fragments of each gene using suitable restriction enzymes, a time consuming and laborious approach. In addition, there are few VIGS vectors available for soybean, and the first virus developed as a soybean VIGS vector, *Bean pod mottle virus* (BPMV), is not known to occur outside North America. The original BPMV VIGS vector placed the insert as an in–frame translational fusion with the viral polyprotein (Zhang and Ghabrial, 2006), and required *in vitro* RNA transcription, two significant restrictions for high throughput usage. Later selection of a mild strain of BPMV and transcription *in planta* from the CaMV 35S promoter and plasmid DNA inoculation increased the utility for VIGS, but VIGS constructs were still required to be inserted in–frame with the polyprotein (Zhang *et al*., 2009). A further adaptation allowed insertion into the 3' non–coding region of the 35S–driven vector (Zhang *et al*., 2010), but still required independent insertion of individual gene constructs. Recently, Nam *et al*. (2012) developed and applied a stable new VIGS vector for soybean. Here, we have modified that vector for increased efficiency of screening gene function in *Glycine max*, through introduction of the Gateway system to allow high–throughput insertion of RNAi constructs in order to identify gene function. In addition to SYCMV we have tested the Gateway system for other vectors such as AltMV and TRV (Ko *et al* unpublished), and this system worked in all tested vectors. Therefore, the newly developed methods introduced in this research could be applied for identifying new gene function in other species. Using the SYCMV VIGS system we have identified several candidate genes controlling growth, and confirmed that our newly develop vectors can be applied to investigate gene function of several crop plants.

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REFERENCES

Baulcombe, D. 2004. RNA silencing in plants. *Nature*. **431**: 356–363. doi: 10.1038/nature02874

Baulcombe, D. C. 1999. Fast forward genetics based on virus–induced gene silencing. *Curr Opin Plant Biol*. **2**: 109–113. doi: 10.1016/s1369–5266(99)80022–3

Brigneti, G., O. Voinnet, W. X. Li, L. H. Ji, S. W. Ding and D. C. Baulcombe 1998. Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *EMBO J*. **17**: 6739–6746. doi: 10.1093/emboj/17.22.6739

Burch–Smith, T. M., J. C. Anderson, G. B. Martin and S. P. Dinesh–Kumar 2004. Applications and advantages of virus–induced gene silencing for gene function studies in plants. *Plant J*. **39**: 734–746. doi: 10.1111/j.1365–313X.2004.02158.x

Cakir, Cahid, Megan E. Gillespie and Steven R. Scofield 2010. Rapid Determination of Gene Function by Virus–induced Gene Silencing in Wheat and Barley. *Crop Sci*. **50**: S–77–S–84. doi: 10.2135/cropsci2009.10.0567

Domier, L. L., H. A. Hobbs, N. K. McCoppin, C. R. Bowen, T. A. Steinlage, S. Chang, Y. Wang and G. L. Hartman 2011. Multiple loci condition seed transmission of *soybean mosaic virus* (SMV) and SMV–induced seed coat mottling in soybean. *Phytopathology* **101**: 750–756. doi: 10.1094/phyto–09–10–0239

Goldberg T., Hamp T., Rost B. 2012. LocTree2 predicts localization for all domains of life. *Bioinformatics* **28**: i458–i465

Hamp T., Kassner R., Seemayer S., Vicedo E., Schaefer C., Achten D., Auer F., Boehm A., Braun T., Hecht M., Heron M., Honigschmid P., Hopf TA., Kaufmann S., Kiening M., Krompass Denis., Landerer C., Mahlich Y., Roos M., Rost B. 2013. Homology–based inference sets the bar high for protein function prediction. *BMC Bioinformatics* (Suppl. Automated Function Prediction Meeting 2011). **14**: Suppl. 3: S7.

Harper, M. E., E. Glynne–Jones, L. Goddard, V. J. Thurston and K. Griffiths 1996. Vascular endothelial growth factor (VEGF) expression in prostatic tumours and its relationship to neuroendocrine cells. *Br J Cancer*. **74**: 910–916.

Hisano, H., S. Sato, S. Isobe, S. Sasamoto, T. Wada, A. Matsuno, S. Tabata 2007 Characterization of the soybean genome using EST–derived microsatellite markers. *DNA research*, **14**(6): 271–281.

Johanson U, West J, Lister C, Michaels S, Amasino R, Dean C. 2000. Molecular analysis of FRIGIDA, a major determinant of natural variation in Arabidopsis flowering time. *Science* **290**(5490): 344–347.

Lim, H. S., A. M. Vaira, L. L. Domier, S. C. Lee, H. G. Kim and J. Hammond 2010. Efficiency of VIGS and gene expression in a novel bipartite potexvirus vector delivery system as a function of strength of TGB1 silencing suppression. *Virology*. **402**: 149–163. doi: 10.1016/j.virol.2010.03.022

Nam, M., J. S. Kim, S. J. Park, C. Y. Park, J. S. Lee, H. S. Choi, J. S. Kim, H. G. Kim, S. Lim, J. S. Moon and S. H. Lee 2012. Biological and molecular characterization of Soybean yellow common mosaic virus, a new species in the genus Sobemovirus. *Virus Res*. **163**: 363–367. doi: 10.1016/j.virusres. 2011.08.005

Nam, M., S. M. Kim, L. L. Domier, S. Koh, J. K. Moon, H. S. Choi, H. G. Kim, J. S. Moon and S. H. Lee 2009. Nucleotide sequence and genomic organization of a newly identified member of the genus Carmovirus, *soybean yellow mottle mosaic virus*, from soybean. *Arch Virol*. **154**: 1679–1684. doi: 10.1007/s00705–009–0480–z

Rost B., Fariselli P., Casadio R. 1996. Topology prediction for helical transmembrane proteins at 86% accuracy. *Protein Sci*. **5**: 1704–1718

Scofield, S. R. and R. S. Nelson 2009. Resources for virus–induced gene silencing in the grasses. *Plant Physiol*. **149**: 152–157. doi: 10.1104/pp.108.128702

Seunghee, Cho, Kim Jungkyu, Li Meijia, Seo Eunyoung, Lim Seungmo, Hong Seok Myeong, Moon Jae Sun, Hammond John and Lim Hyoun–Sub 2013. Occurrence of Three Major Soybean Viruses, *Soybean mosaic virus*, *Soybean yellow mottle mosaic virus* and *Soybean yellow common mosaic virus* Revealed by a Nationwide Survey of Subsistence Farming Soybean Fields. *Research in Plant Disease*. **19**: 319–325.

Voinnet, O., C. Lederer and D. C. Baulcombe 2000. A viral movement protein prevents spread of the gene silencing signal in *Nicotiana benthamiana*. *Cell*. **103**: 157–167.

Zhang C, Bradshaw JD, Whitham SA, Hill JH. 2010. The development of an efficient multipurpose bean pod mottle virus viral vector set for foreign gene expression and RNA silencing. *Plant Physiol*. **153**(1):52–65. doi: 10.1104/pp.109.151639.

Zhang, C. and S. A. Ghabrial 2006. Development of Bean pod mottle virus–based vectors for stable protein expression and sequence–specific virus–induced gene silencing in soybean. *Virology*. **344**: 401–411. doi: 10.1016/j.virol.2005.08.046

Zhang C, Yang C, Whitham SA, Hill JH. 2009. Development and use of an efficient DNA–based viral gene silencing vector for soybean. *Mol Plant Microbe Interact*. **22**(2):123–131. doi: 10.1094/MPMI–22–2–0123.