Construction of a recombinant vector containing SAI gene using Gateway-based RNAi system: a preliminary study in the development of high sucrose sugarcane

T Hadiarto*, E I Riyanti, E Listanto and A Polosoro

Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development, Indonesian Agency for Agricultural Research and Development
Jl. Tentara Pelajar 3A, Bogor 16111, Indonesia

*Email: totohadiarto@pertanian.go.id

Abstract. Recombinant plasmid construction that produces hairpin molecules for gene knockdown is not an easy method. Gateway cloning system is a technology to transfer DNA fragments utilizing clonase and specific sites. This study aimed to construct RNAi expression vector containing soluble acid invertase (SAI) gene using Gateway system to produce high-sugar content sugarcane. PCR was conducted to generate specific SAI fragment using cDNA template from two sugarcane varieties: Bululawang and PSJK922. Of the two specific primer pairs designed, only one pair amplified the SAI 161 bp gene fragment. Amplification occurred in both cDNA samples. TA (Thymine-Adenine pair) cloning was performed with pCR8/GW/TOPO plasmid and a clonase reaction was carried out to insert the specific fragments into the pHELLSGATE 8 hairpin plasmid. The results showed that two of the four colonies analyzed by associated restriction enzymes had plasmid pHELLSGATE8::hpSAI that contained a hairpin component of the SAI fragment. One attempt to construct the hairpin RNA using Gateway system in this study indicates the easiness of the system when compared to traditional cloning. The DNA sequence in the hairpin portion of this plasmid will be analysed to confirm SAI gene fragment, and it will be followed by transformation of sugarcane and SAI characterization.

1. Introduction
In 2018, as many as 70 countries have cultivated and/or imported genetically modified (GM) crops such as non-bruising, non-browning, late blight resistant potatoes, insect resistant and drought tolerant sugarcane, non-browning apples, and herbicide resistant soybeans [1,2]. Four major worldwide-cultivated GM crops include soybean, maize, cotton, and rapeseed. The growingly adopted GM crops contributed to overcome food resource problems that may arise from population growth, climate change and environmental destructions [2].

GM crops development has mainly been supported by the advancing technology of genetic modification. Gene silencing plays as important roles as gene overexpression or inducible gene expression in transgenic plant construction. Gene silencing naturally occurs as eukaryotic defense mechanism against incoming pathogenic invasion [3]. It involves the production of double-stranded RNA (dsRNA) or hairpin-structured RNA (hpRNA) that would be degraded inside the cell or cause inhibition of protein synthesis [4,5]. Gene silencing technology has been used to study the function of a gene by producing cells with the absence of its products [6]. In plants, the technology has been...
applied to explore plant genomes [7]. In addition, construction of plants with specific undesired molecules (such as allergens or toxins) inhibition has been developed, for example genetically modified apples with reduced clinical allergenic protein Mal d-1 [8], cottonseed with ultra-low gossypol content [9], and low-linolenic acid soybean [10,11]. Furthermore, gene silencing has been used to develop plants with pathogen resistant characteristic, such as foliar pathogen resistant in soybean [12], leaf rust fungus resistant in wheat [13], and yellow leaf virus resistant in tomato [14].

The underlying mechanisms of gene silencing was initially observed in the virus-induced genetically-engineered plant with the suggestion of antisense RNA generation to suppress expression [15]. Information had grown ever since, producing more understanding on the mechanisms and more advantageous plants were engineered [16]. The unraveling of gene silencing mechanisms step by step produces significant advancement in gene silencing technology including RNA interference (RNAi) or hpRNA, small RNA (sRNA) or small-interfering RNA (siRNA), microRNA (miRNA) and trans-acting siRNA (tasiRNA) technologies [17-19].

In conjunction with the development of gene silencing technology, cloning methods have also demonstrated excellent advancement. TA (Thymine-Adenine pair) cloning has made cloning of PCR products simpler and more efficient. Vectors with high efficiency has been developed for TA cloning and blunt-end cloning [20]. Additional contribution from Gateway cloning system has helped characterization and functional studies in many research activities [21].

Gateway cloning is considered simple and efficient cloning system since several often-problematic and/or laborious experimental steps are simplified by the utilization of an enzyme called clonase. Clonase precisely recognizes specific att site in the vector and generates recombinations [22]. There are two types of clonase enzymes: LR clonase and BP clonase. LR clonase specifically recognizes attL and attR sites and recombinates them to produce attB and attP sites, while BP clonase acts vice versa (figure 1).

![Figure 1. Schematic diagram to illustrate how LR and BP clonase enzymes work in the Gateway cloning system [23].](image1)

Two types of plasmids/vectors used in the system: entry clone and destination vectors. The entry clone is a recombinant plasmid that contains the DNA fragment of interest. Generation of entry clone can be performed by three different methods: TA cloning using plasmid such as pENTR and pCR8 [24], by clonase reaction using appropriate donor vector such as pDONR [25], or by more conventional method of restriction cloning using restriction enzyme and ligase with a donor vector.

Once inside the the entry clone, the DNA fragment of interest can be transferred into preferred destination vector by means of clonase recombination. There are many choices of destination vectors that have been developed [25]. To choose the destination vector, there are several factors to be considered, such as the type of organism to use (E. coli, mammalian cells, yeast, etc), the expression level and more importantly, the purpose of the research. Several examples of destination vectors are pDEST, pYES2-DEST52, pLenti and pHELLSGATE [26-28].

pHELLSGATE vectors were constructed by Helliwell research groups [29,30]. The vectors harness the strength of Gateway system in producing hpRNA. The combination of pHELLSGATE and Gateway system enables the construction of hpRNA in a single step [31]. The vectors consist a pair of replaceable ccdB genes for hairpin formation with PDK intron loop, and the genes are which are enclosed by att clonase recognition sites for DNA fragments replacement (Fig. 2). The effectiveness of these vectors have been claimed to be as good as those generated by traditional cloning methods [31].
Figure 2. Map of pHELLSGATE8 as the recipient vector. The insert within attL in the entry clone (in this case the SAI gene fragment) will be transferred to the attR in the recipient vector by LR clonase enzyme (https://www.snapgene.com/resources/plasmid-files/?set=plant_vectors&plasmid=pHELLSGATE_8).

To date, the Gateway technology has developed into more sophisticated technology that enables cloning and fragment construction methods more convenient. Choices of both entry and destination vectors have increased, hence transfer of DNA fragments between vectors are more accessible. They are being developed not only by private companies, but also national agencies such as CSIRO, Australia. A dual-site cloning system has been developed to clone two genes at the same time [32]. Other simultaneous integration of multiple DNA fragment in Gateway system has been reported [29-31], including assembly kit made of modular plasmid for expression/silencing study [32,33].

In this research an expression vector with Gateway cloning system approach was generated. The expression vector produced by this technology is expected to carry SAI gene fragment inserts that will form hpRNA inside transformed plant cells. SAI (soluble acid invertase) is one of the two known acid invertases that play important roles in sucrose production [37]. High invertase concentration in sugarcane plant has been related with lower sucrose level [38]. Therefore, RNAi system is adopted with the support of Gateway cloning system to generate recombinant vector containing SAI gene fragment. The RNAi system is proposed to reduce SAI concentration in sugarcane.

This is a preliminary research to generate sugarcane with higher sucrose level as a final product. It is expected that the product will lead to self-sufficient sugar production therefore the import of sugar in Indonesia can be reduced, thus economically advantageous.
2. Materials and Methods

2.1. Sugarcane RNA isolation
Two sugarcane varieties were used as the source of RNA; PSJK922 and Bululawang. Total RNA from 3-month old sugarcane plantlets grown on MS media tissue culture was isolated using the RNeasy Plant Mini Kit (Qiagen, USA), following the manufacturer’s instruction. One hundred mgs of sugarcane leaves were ground to fine powder in cold sterile mortars using liquid nitrogen. Then the leaf cell was broken down and homogenized with the lysis buffer available in the kit. The next step was to follow the instructions from the kit. In the final stage, as much as 50 μl of sterile MilliQ water was used to elute RNA from the column.

2.2. Sugarcane cDNA generation
Total RNA template was used to generate cDNA by means of Reverse-transcription Polymerase Chain Reaction (q-PCR) using the SuperScript™ II Reverse Transcriptase (Invitrogen, USA). The reaction mixture consisted of 5 μg of total RNA, 1 μl oligo (dT)12-18, 10 mM dNTP and sterile MilliQ water to produce the final volume of 12 μl. This mixture was then incubated at 65°C for 5 minutes, after which it was quickly transferred to ice, and centrifuged to precipitate the solution. Four μl of 5x First strand buffer, 2 μl of 0.1 M DDT and 1 μl RNase OUT (Invitrogen, USA) were added to the mixture, slowly stirred and then incubated at 42°C for 2 minutes. As much as 1 μl SuperScript™ II RT was then added, and sterile MilliQ water was added so that the total volume became 20 μl. The solution was incubated at 42°C for 50 minutes, after which the reaction was stopped by incubating the solution at 75°C for 15 minutes. The first cDNA strand product was then used to generate the desired fragments by PCR.

2.3. PCR
PCR was performed to amplify SAI gene fragments from cDNA. SAI gene sequences can be accessed from the site http://www.ncbi.nlm.nih.gov/ with GenBank ID: AF062735.1. The sequence of SAI gene was used to design forward and reverse (specific) primers. The primers were designed to obtain two amplicons with the sizes of 161 bp and 171 bp. The design was done manually by paying attention to the important elements for successful PCR and analyzes for the possibility of primer dimer was performed using the Multiple Primary Analyzer (https://www.thermofisher.com/id/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html). The results of primer design, including the primer sequences and amplicon sizes, were shown in table 1.

PCR mix was prepared by adding up 10X PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl (5 μl), 50 mM MgCl2 (1.5 μl)], 10 mM dNTP Mix (1 μl), specific forward primers (10 μM, 1 μl), specific reverse primer (10 μM, 1 μl), Taq DNA polymerase (5 U / μl, 0.4 μl), 2 μl cDNA and sterile MilliQ water to obtain a total volume of 50 μl into a PCR tube. The PCR was run under the condition of: the first stage of denaturation at 94°C for 2 minutes, followed by a 35 PCR cycles with an initial denaturation stage at 95°C for 30 seconds, primer attachment at a temperature between 50-60°C (adjusted for primers) for 30 seconds and extension at 72°C for 2 minutes. The PCR cycle ends at 75°C for 5 minutes. PCR products were run on agarose electrophoresis using 1% agarose gel in 1x Tris Acid EDTA (TAE). The results were visualized using Chemidoc gel system (Biorad).

2.4. Cloning of SAI gene fragment
TA cloning was performed to insert the PCR generated SAI gene fragment. The gene fragment was ligated to the pCR8 / GW / TOPO vector (Fig. 3) by adding 0.5-1 μl PCR product, 1 μl salt solution, 1 μl vector, and sterile water until the final volume became 6 μl, following the manufacturer’s instruction. After 5 minutes at room temperature incubation, the solution was chilled in ice and ready to be transformed into competent cells. The recombinant entry clone which is plasmid pCR8 / GW / TOPO that was successfully ligated with SAI gene fragments is called pCR8::hpSAI.
Figure 3. Map of TA plasmid used to clone SAI gene fragment. The presence of attL1 and attL2 regions are essential to move the fragment into other Gateway-related vectors (https://www.thermofisher.com/).

Transformation into competent E. coli cells was carried out by the heat shock method [39]. A total of 2 µl cloning result was used to transform 50 µl one shot E. coli competent cells strain recA, endA TOP10 (Invitrogen, USA). After heat shock and incubation on ice, the mixture was added with 250 µl LB broth and continued with incubation at 37°C for 1 hour. The bacterial cells were then precipitated by centrifugation. A total of 200 µl of liquid media was discarded and the remainder of the media and cell sediment was shaken and then poured evenly on LB media containing 100 µg/ml spectomycin. The cell culture was incubated at 37°C overnight. Six colonies were grown on 3 ml liquid LB containing spectomycin antibiotic for plasmid extraction. The liquid culture was incubated at 37°C on a shaker at 250 rpm overnight.

2.5. Plasmid isolation
Plasmid was isolated using QIAprep Spin miniprep kit (Qiagen, USA). All steps were carried out following the instruction manuals. Isolated plasmid was run on agarose gel electrophoresis.

2.6. Construction of pHellsgate containing SAI hairpin construct
The insertion of SAI gene fragments into the pHellsgate 8 expression vector (figure 2) was performed using a Gateway cloning system through the LR reaction. A 2 µl pCR8::hpSAI (containing 100-300 ng DNA) was added with 2 µl recipient vector, pHellsgate 8 vector (150ng / µl), 5x LR clonase reaction buffer as much as 4 µl, TE buffer (pH 8.0) 8 µl in a 1.5 ml eppendorf tube. A total of 4 µl LR clonase enzyme was added to the initial reaction and then vortexed 2 times quickly, followed by rapid centrifugation to collect the solution at the bottom of the tube. The solution was then incubated at 25°C for 60 minutes. Two µl Proteinase K was added to the reaction and incubated at 37°C for 10 minutes to stop the reaction. The results of LR reaction was transformed as before while replacing the spectomycin with 100 µg/ml kanamycin, and followed by transformant analysis using restriction digestions.

2.7. Restriction Digestion
2 µl of plasmid DNA (1 µg/µl) was mixed with 1 µl of restriction enzyme (XhoI or XbaI), 2 µl of 10x buffer and 15 µl of sterile ddH₂O. After heating at 37°C for 1 hour, the sample was analyzed on agarose gel electrophoresis.

3. Results and Discussion
3.1. SAI gene fragment amplification and Entry clone generation
Amplification of DNA fragments from the SAI gene was carried out to generate RNAi constructs through the Gateway system. The cDNA sequence of SAI gene (GenBank ID: AF062735.1) from the existing gene bank database is used as a reference template for making specific forward and reverse
primers. From the sequence, two primer sets were designed to amplify SAI gene fragments. Details on the primer sequences, positions and amplicon sizes are shown in table 1 and figure 4.

Table 1. PCR primers designed to generate the SAI gene fragment.

| Name       | Sequence               | Size (nt) | Amplicon size(bp) | Primer Dimer |
|------------|------------------------|-----------|-------------------|--------------|
| SAI Gw F1  | CGTTCCCGTGAGCAAT       | 17        | 161               | -            |
| SAI Gw R1  | CTTGTTGCCCCAGATGGC     | 18        |                   |              |
| SAI Gw F2  | GTGCCCGACCAGTGTTA      | 17        | 171               | v            |
| SAI Gw R2  | CTTGGTCCAGTGGGAGC      | 19        |                   |              |

nt = nukleotide; bp = base pair; - = absence; v = presence

Figure 4. Schematic diagram showing the positions of primers used in the cDNA sequence of SAI (orange bar) and expected amplicon (blue bar) sizes. F1 dan R1 = forward and reverse primers for 161-bp SAI fragment. F2 dan R2 = forward and reverse primers for 171-bp SAI fragment.

The cDNA synthesis was carried out by qPCR using the total RNA source from sugarcane varieties PSJK922 and Bululawang. The two pairs of primers that have been designed were used to amplify SAI gene fragments from the cDNA material. The results of the amplification of SAI gene fragments with the two pairs of primers shown that the primer set 1 did amplify DNA pieces of SAI genes of about 161 bp both from cDNA sugarcane variety PSJK922 or Bululawang varieties (figure 5). The second primer set did not amplify the estimated SAI gene fragment, probably due to the presence of a dimer primer at the time of PCR process.

Figure 5. The results of PCR amplification of SAI gene fragments using two sets of primers. B1 = Bululawang template using primer set 1, B2 = Bululawang template using primer set 2, P1 = PSJK922 template using primer set 1, P2 = PSJK922 template using primer set 2, M = 1kb plus DNA ladder (Invitrogen, USA).

The amplicon (161bp) of the Bululawang (B1) template was chosen for the pCR8/GW/TOPO vector transformation. This TA plasmid has an attL DNA sites that are recognized by the clone enzyme to create recombinant expression vectors. There are about 100 colonies growing on the
selection media from the transformation. Six colonies were chosen to confirm the presence of the inserts in pCR8 vector. The plasmids were digested with EcoRI.

The results showed that all digested plasmids contain inserts with a size of about 180bp which is the total size of the SAI gene fragment resulted from the PCR and pCR8 flanking region (figure 6). These recombinant entry clone is named pCR8::hpSAI.

**Figure 6.** Confirmation of cloning of SAI gene fragment in pCR8 entry vector by EcoRI restriction digestion on 6 plasmid samples. D= EcoRI digested, U= undigested plasmid, M = wide range 50-10,000 bp DNA ladder (Takara, Japan).

3.2. Construction of pHELLSGATE8-SAI clone

Gateway technology was adopted as an attempt to silence SAI gene. The technology provides simple cloning method of hpRNA in the RNAi system. The mixture of pCR8::hpSAI clone and pHELLSGATE8 (provided by CSIRO, Australia) was aimed to produce pHLLSAGATE expression clone which contained the SAI gene fragment for hpSAI. Figure 7 provides a systematic diagram to illustrate the process using the Gateway technology.

LR clonase and its buffer were added to the mixture of pCR::hpSAI clone and pHELLSGATE8 vector. LR clonase would recombine attL and attR sites and replace the inserts of ccdB gene with SAI gene fragment. The final products are two plasmids consisting of pCR8 containing ccdB gene and pHELLSGATE containing SAI fragments (or pHELLSGATE::hpSAI). Following transformation, when the cells are grown in selective media containing appropriate amount of antibiotic kanamycin, only transformed cells containing pHELLSGATE plasmid should grow.
Figure 7. Schematic diagram of Gateway cloning reaction involving pCR8::hpSAI and pHELLSGATE8 plasmids to generate pHELLSGATE8::hpSAI clone that has hairpin construct for RNAi.

In order to confirm the success of pHELLSGATE8 expression clone construction, plasmids extracted from the transformants were digested with XhoI and XbaI. Theoretically XhoI cuts out the first fragment in the plasmid after CaMV 35S promoter to give the predicted size of 1430 bp fragment in the original plasmid or 1307 bp in the recombinant pHELLSGATE8::hpSAI, while XbaI cuts the second fragment upstream the NOS promoter (figure 2) to give the predicted size of 1427 bp fragment in the original plasmid or 1304 bp in the recombinant pHELLSGATE8::hpSAI. The results of the digestion demonstrate that 1470 pb fragments were produced (figure 8). This fragment size is different from that obtained from original pHELLSGATE8 digestion. This indicates that the original fragment (ccdB gene) within the attR regions were replaced successfully by other fragment. The 1470 bp fragment size gave strong indication that this replacing fragment is SAI gene fragment. These results need to be sequenced to confirm the presence and the direction of the SAI gene fragments in the expression clone.

Figure 8. Confirmation of SAI gene fragment insert inside pHELLSGATE expression vector by using XhoI and XbaI digestion. Or=original pHELLSGATE containing ccdB gene; 1-4: plasmid from LR clonase reaction and were expected to generate RNAi construct with SAI gene fragment; M= 1kb plus DNA ladder (New England Biolabs, USA).
Overall, the process of cloning conducted with the Gateway system has been simple and straightforward. Two out of four plasmids analyzed gave positive result of the probable generation of pHELLSGATE8::hpSAI expression clone (figure 8). The construction of hairpin plasmids using conventional cloning would normally take longer and more challenging. Four restriction digestions are required to clone a short fragment into a hpRNA plasmid such as pHANNIBAL [28]. Table 2 compares the steps of experiments in the construction of hpRNA plasmids using traditional cloning and Gateway system [18,28].

Table 2. Comparisons of experimental steps between conventional cloning and Gateway cloning for the construction of hpRNA plasmid [18,28,33].

| Steps | Conventional Cloning | Gateway Cloning |
|-------|-----------------------|-----------------|
| 1     | Generation of PCR product | Generation of PCR product |
| 2     | Entry cloning | Entry cloning |
|       | - TA cloning in appropriate TA plasmid or restriction enzyme cloning | - TA cloning in appropriate TA plasmid or restriction enzyme cloning |
| 3     | hpRNA cloning | hpRNA cloning |
|       | - Four restriction digestions* | - Clonase reaction |
|       | - One to two ligation* | - E. coli transformation |
|       | - E. coli transformation | |

* indicates higher difficulty level and more time consumption

In the conventional method, the digestion for preparation of both the plasmid and the DNA insert is more difficult to do and consuming more time. The difficulty level increases with ligation step to place the insert in the correct orientation. This construction of hairpin RNA of SAI gene fragment using Gateway system have given simpler and more efficient method when compared to conventional cloning. The consideration to be made is the cost of the clonase enzyme which is more expensive when compared to Taq polymerase, for instance. However, less time consumption and more efficiency given by Gateway method should be considered as compensation, thus can neglect the expense of this system.

4. Conclusion
Gene silencing construct employing RNAi construct to target SAI has been developed by means of Gateway cloning system. The process of construction of the clone has been simple and efficient. The pHELLSGATE8::hpSAI expression clone that has been generated contained two identically opposite 161 bp SAI fragment that will form a hairpin upon transcription. The fragments need to be confirmed by DNA sequencing. Once confirmed, the clone will be utilized in transformation to develop high sucrose sugarcane.

References
[1] ISAAA 2018 Global status of commercialized biotech/GM crops in 2018 ISAAA Br. 54 ISAAA Ithaca, NY
[2] Matsuo A, Matsushita K, Fukuzumi A, Tokumasu N, Yano E, Zaima N and Moriyama T 2020 Foods 9 522
[3] Guo Q, Liu Q, Smith N A, Liang G and Wang M B 2016 Genomics 17 476–89
[4] Kim D H and Rossi J J 2008 Biotechniques 44 613–6
[5] Leirdal M and Sioud M 2002 duplexes 295 744–8
[6] Baykal U and Zhung Z 2010 Gene Silencing: Theory, Techniques and Applications ed A J Catalano (Nova Science Publishers, Inc.) pp 255–69
[7] Waterhouse P and Helliwell C 2003 Exploring plant genomes by RNA-induced gene silencing *Nat. Rev. Gen.* 4 29–38
[8] Dubois A E J, Pagliarani G, Brouwer R M, Kollen B J, Dragsted L O and Eriksen F D 2015 *Allergy* 70 1406–12
[9] Rathore K S, Pandeya D, Campbell L M, Thomas C, Puckhaber L, Stipanovic R D, Thenell J S, Hague S, Hake K, Puckhaber L, Stipanovic R D, Thenell J S and Hague S 2020 *CRC. Crit. Rev. Plant Sci.* 39 1–29
[10] Flores T, Karpova O, Su X, Zeng P, Bilyeu K, Sleper D, Nguyen H and Zhang Z 2008 *Transgenic Res.* 17 839–50
[11] Held J P, Carrero-Colon M and Hudson K A 2019 *Agrosyst. Geosci. Env.* 2 1–4
[12] Rathore K S, Pandeya D, Campbell L M, Thomas C, Puckhaber L, Stipanovic R D, Thenell J S, Hague S, Hake K, Puckhaber L, Stipanovic R D, Thenell J S and Hague S 2020 *CRC. Crit. Rev. Plant Sci.* 39 1–29
[13] Panwar V, McCallum B and Bakkeren G 2013 *Plant Mol. Biol.* 81 199–260
[14] Luna A P, Morilla G, Voinnet O and Bejarano E R 2012 *Mol. Plant-Microbe Interact.* 25 1294–306
[15] Inouye M 1988 *Gene* 72 25–34
[16] Hofgen R and Willmitzer L 1992 *Plant Sci.* 87 45–54
[17] Cheng C, Zhang Y, Yang J and Zhong Y 2017 *J. Hortic. Sci. Biotechnol.* 92 465–74
[18] Eamens A, Wang M, Smith N A and Waterhouse P M 2008 *Plant Physiol.* 147 456–68
[19] Meyers B C and Axtell M J 2019 *Plant Cell* 31 1206–7
[20] Kandoth P K, Heinz R, Yeckel G, Gross N W, Juvale P S, Hill J, Whitham S A, Baum T J and Mitchum M G 2013 *BMC Res Notes* 6 255
[21] Panwar V, McCallum B and Bakkeren G 2013 *Plant Mol. Biol.* 81 199–260
[22] Luna A P, Morilla G, Voinnet O and Bejarano E R 2012 *Mol. Plant-Microbe Interact.* 25 1294–306
[23] Inouye M 1988 *Gene* 72 25–34
[24] Hofgen R and Willmitzer L 1992 *Plant Sci.* 87 45–54
[25] Panwar V, McCallum B and Bakkeren G 2013 *Plant Mol. Biol.* 81 199–260
[26] Luna A P, Morilla G, Voinnet O and Bejarano E R 2012 *Mol. Plant-Microbe Interact.* 25 1294–306
[27] Inouye M 1988 *Gene* 72 25–34
[28] Hofgen R and Willmitzer L 1992 *Plant Sci.* 87 45–54
[29] Panwar V, McCallum B and Bakkeren G 2013 *Plant Mol. Biol.* 81 199–260
[30] Luna A P, Morilla G, Voinnet O and Bejarano E R 2012 *Mol. Plant-Microbe Interact.* 25 1294–306
[31] Inouye M 1988 *Gene* 72 25–34
[32] Hofgen R and Willmitzer L 1992 *Plant Sci.* 87 45–54
[33] Panwar V, McCallum B and Bakkeren G 2013 *Plant Mol. Biol.* 81 199–260
[34] Luna A P, Morilla G, Voinnet O and Bejarano E R 2012 *Mol. Plant-Microbe Interact.* 25 1294–306
[35] Inouye M 1988 *Gene* 72 25–34
[36] Hofgen R and Willmitzer L 1992 *Plant Sci.* 87 45–54
[37] Panwar V, McCallum B and Bakkeren G 2013 *Plant Mol. Biol.* 81 199–260
[38] Luna A P, Morilla G, Voinnet O and Bejarano E R 2012 *Mol. Plant-Microbe Interact.* 25 1294–306
[39] Sambrook J F and Russell D 2001 *Molecular Cloning: A Laboratory Manual (3-volume set)*