An Improved Single-Step Cloning Strategy Simplifies the *Agrobacterium tumefaciens*-Mediated Transformation (ATMT)-Based Gene-Disruption Method for *Verticillium dahliae*

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Accepted for publication 14 January 2016.

ABSTRACT

Wang, S., Xing, H., Hua, C., Guo, H.-S., and Zhang, J. 2016. An improved single-step cloning strategy simplifies the *Agrobacterium tumefaciens*-mediated transformation (ATMT)-based gene-disruption method for *Verticillium dahliae*. *Phytopathology* 106:645-652.

The soilborne fungal pathogen *Verticillium dahliae* infects a broad range of plant species to cause severe diseases. The availability of *Verticillium* genome sequences has provided opportunities for large-scale investigations of individual gene function in *Verticillium* strains using *Agrobacterium tumefaciens*-mediated transformation (ATMT)-based gene-disruption strategies. Traditional ATMT vectors require multiple cloning steps and elaborate characterization procedures to achieve successful gene replacement; thus, these vectors are not suitable for high-throughput ATMT-based gene deletion. Several advancements have been made that either involve simplification of the steps required

for gene-deletion vector construction or increase the efficiency of the technique for rapid recombinant characterization. However, an ATMT binary vector that is both simple and efficient is still lacking. Here, we generated a USER-ATMT dual-selection (DS) binary vector, which combines both the advantages of the USER single-step cloning technique and the efficiency of the herpes simplex virus thymidine kinase negative-selection marker. Highly efficient deletion of three different genes in *V. dahliae* using the USER-ATMT-DS vector enabled verification that this newly-generated vector not only facilitates the cloning process but also simplifies the subsequent identification of fungal homologous recombinants. The results suggest that the USER-ATMT-DS vector is applicable for efficient gene deletion and suitable for large-scale gene deletion in *V. dahliae*.

Additional keyword: HSVtk.

Verticillium dahliae is a soilborne fungal pathogen that infects a broad range of dicotyledonous plant species causing economically important vascular wilt diseases (Armengol and Berbegal 2005; Gao et al. 2010; Klosterman et al. 2009; Pegg and Brady 2002; Vallad and Subbarao 2008). Infected plants show severe disease symptoms such as leaf wilting, plant stunting, and vascular discoloration, which vary depending on host type (Klimes et al. 2015). Due to host vascular colonization and long-term soil viability, *Verticillium* diseases are very difficult to control and represent major threats to commercial crops, especially in most cotton-growing areas.

The molecular mechanisms underlying *Verticillium* infection remain largely unclear. Thus far, very few genes have been reported to be involved in the spore development and virulence of *Verticillium* spp. (Klimes et al. 2015). As in many filamentous fungi, gene functional analysis in *Verticillium* spp. mainly relies on *Agrobacterium tumefaciens*-mediated transformation (ATMT)-based gene-disruption strategy (Klimes and Dobinson 2006; Wang et al. 2004; Zhou et al. 2012). Generation of a gene-deletion construct and selection of recombinants that have undergone gene replacement are two core steps that determine the efficiency and throughput of ATMT-based gene-deletion strategy. This method

has been optimized either by simplifying the vector construction procedure or by improving the selection efficiency of fungal recombinants (Frandsen et al. 2008; García-Pedrajas et al. 2008; Khang et al. 2005; Paz et al. 2011).

Generation of a gene-deletion construct requires the cloning of a homologous recombination cassette, which contains a positive selection (PS) marker (usually hygromycin B phosphotransferase [HPT]) bordered by upstream and downstream homologous sequences flanking the target gene into a binary vector. It typically requires multiple cloning steps to sequentially assemble three fragments, including the upstream flanking homologous sequence, the PS marker gene, and the downstream flanking homologous sequence, into the binary vector backbone. Traditional methods for assembly of the three fragments include sequential restriction enzyme digestion followed by ligase-dependent cloning and Gateway cloning technology (García-Pedrajas et al. 2008).

Several one-step cloning techniques have recently been introduced to facilitate vector construction, including the In-Fusion construction strategy, the one-step construction of *Agrobacterium* recombination-ready plasmids (OSCAR) method, and the USER cloning technique. The In-Fusion construction strategy was developed based on the 3' to 5' single-strand exonuclease activity of poxvirus DNA polymerase (Frandsen et al. 2008, 2011). This method requires the introduction of four unique 10- to 15-bp extensions, which are identical to the flanking sequences of restriction enzyme recognition sites in the vector, into polymerase chain reaction (PCR) primer pairs for homologous sequence amplification (Frandsen et al. 2008, 2011). Each end of the two PCR-amplified homologous sequences thus carries a unique 10- to 15-bp extension. The binary vector, containing the HPT selection marker, is digested by the corresponding restriction enzymes to generate both a backbone fragment

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*The e-Xtra logo stands for "electronic extra" and indicates that two supplementary figures and one supplementary table are published online.

<http://dx.doi.org/10.1094/PHYTO-10-15-0280-R>
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and an HPT fragment. These two fragments are incubated together with the above-described PCR-amplified homologous sequences and In-Fusion enzyme to generate the gene-deletion construct. Although the In-Fusion construction strategy offers one-step cloning, it produces correctly assembled constructs at a relatively low frequency (Frandsen et al. 2008).

The OSCAR method generates gene-deletion construct based on Gateway technology (Paz et al. 2011). This method requires the construction of two cloning vectors. One is a T-DNA binary vector carrying two different attB recombination sites (attB1 and attB2), and the other is a marker vector containing the HPT gene bordered by two additional attB recombination sites (attB3 and attB4). Four unique attB recombination sites corresponding to those present in the T-DNA binary vector and the marker vector are introduced into PCR primer pairs for homologous sequence amplification. Thus, each end of the two PCR-amplified homologous sequences carries a unique attB recombination site. The two vectors together with two PCR-amplified homologous sequences are incubated in a single BP

Clonase reaction to generate the gene-deletion construct. The average correct assembly efficiency of the OSCAR method is 80% (Paz et al. 2011).

The USER cloning technique is another efficient one-step cloning strategy that relies on the combined action of uracil DNA glycosylase, DNA glycosylase, and Endo VII (Frandsen et al. 2008, 2011; Santhanam and Thomma 2013). The vector contains two USER cloning sites (UCS), which can be sequentially cleaved by two restriction enzymes to generate a vector backbone with two unique overhangs (UCS1 and -2) and an HPT gene fragment with two other unique overhangs (UCS3 and -4). Four unique 9-bp extensions, including a deoxyuridine triphosphate, are introduced into PCR primer pairs for homologous sequence amplification. Thus, each end of the two PCR-amplified homologous sequences carries a unique 9-bp extension for USER cloning. The vector backbone and HPT fragments, generated by restriction enzyme digestion, are incubated together with two PCR-amplified homologous sequences and USER enzyme mix to generate the gene-deletion construct. The

Fig. 1. Schematic description for generation of USER *Agrobacterium tumefaciens*-mediated transformation dual-selection (ATMT-DS) vector deployed in this study. The fragment harboring a *Sma*I site was digested by *Hind*III and *Xba*I from pSulPH-EGFP, and cloned into pGKO-Gateway to generate an intermediate vector. HPTBOX plus USER cloning sites were polymerase chain reaction (PCR) amplified from pRF-HU2, and cloned into the intermediate vector to generate pGKO-hygromycin B phosphotransferase (HPT) USER-ATMT-DS vector.

USER technique offers an average correct assembly efficiency of 95% (Frandsen et al. 2008).

Efficient selection of transformants with the correct gene-replacement event is critical for large-scale ATMT-based gene deletion. In the majority of conventional ATMT-PS binary vector-based gene-deletion methods, only the PS marker HPT is used as a selection marker for fungal transformants. In addition to targeted gene-replacement mutants generated from homologous recombination, this method also generates many ectopic transformants via random T-DNA integration rather than successful homologous recombination and requires additional PCR-based screening to identify the correct targeted gene-replacement mutants from numerous HPT-resistant transformants. Therefore, the use of traditional ATMT vectors to obtain gene knockout mutants can be time consuming and labor intensive. Introduction of a second selection marker will greatly facilitate the identification of recombinants that have undergone gene replacement, because gene-replacement mutants carry only the HPT marker gene while ectopic transformants carry both selection markers. The second selection marker could be either positive or negative. The herpes simplex virus thymidine kinase (*HSVtk*) is capable of converting 5-fluoro-2'-deoxyuridine (5FU) to a compound that is toxic to fungi. Correct target gene-replacement mutants carry only the *HPT* resistance gene, whereas ectopic transformants carry both the *HPT* resistance gene and the *HSVtk* gene. *HSVtk* expressed in ectopic transformant is able to convert 5FU in the medium to a toxic compound; thus, *HSVtk* can be used to eliminate ectopic transformants (Khang et al. 2005). This counter-selection against ectopic transformants significantly simplifies the identification of targeted gene-deletion mutants. *HSVtk* was introduced into the ATMT-PS binary vector to generate the ATMT-dual-selection (DS) vector, and this vector is efficient for gene deletion in *Magnaporthe grisea* and *Fusarium oxysporum* (Khang et al. 2005).

Taken together, the one-step cloning ATMT vectors require elaborate characterization procedures to identify gene-replacement recombinants, and the currently available ATMT-DS vectors require multiple cloning steps. Thus, it is necessary to generate a highly efficient ATMT vector which carries both advancements and is suitable for high-throughput gene deletion.

The objective of this study was to integrate the single-step USER cloning technique with the *HSVtk* negative-selection marker to improve the process of gene deletion in *V. dahliae*. *HSVtk* was introduced into a UCS-containing binary vector to generate the USER-ATMT-DS vector for targeted gene-deletion. Successful deletion of *VdGARP1* (VDAG_08781) (Gao et al. 2010) using the USER-ATMT-DS vector verified the efficacy of the modified vector for gene deletion in *V. dahliae*, and two additional genes,

herein. Compared with other ATMT-based gene-deletion approaches, the USER-ATMT-DS method is more simplistic and also more efficient. The results indicate that the USER-ATMT-DS vector is an ideal vector for large-scale gene deletion in *V. dahliae*, and suggest the potential utility of the USER-ATMT-DS vector in other filamentous fungi.

MATERIALS AND METHODS

Fungal and bacterial strains. *V. dahliae* strain 592 (V592) isolated from cotton originated in Xinjiang, China (Gao et al. 2010) was used as recipient strain for gene deletion. *Escherichia coli* strain DH5 α was used to propagate all the plasmids. *A. tumefaciens* strain EHA105 was used for fungal transformation. Potato dextrose agar (PDA) medium was used to culture V592. PDA medium containing 5FU at 20 μ g/ml, hygromycin B at 50 μ g/ml, or sulfonylurea (SUL) at 100 μ g/ml was used to culture *V. dahliae* transformants generated by the USER-ATMT-DS or ATMT-PS-SUL method, as indicated. ATMT-mediated *V. dahliae* transformation was performed as described by Gao et al. (2010).

Vectors and plasmids used in this study. The pGKO-Gateway vector was used as a backbone binary vector. pRF-HU2 was used as template for the amplification of the HPT coding sequence (HPTBOX) plus USER cloning sites. HPTBOX plus USER cloning sites were then cloned into pGKO-Gateway to generate USER-ATMT-DS vector. The upstream and downstream flanking sequences of *VdGARP1*, *VDH1* (VDAG_02273) (Klimes and Dobinson 2006), and *VdNLP2* (VDAG_01995) (Zhou et al. 2012) were PCR amplified from V592 genomic DNA, with the reference genome sequence of *V. dahliae* strain Ls.17 (*VdLs.17*) (Klosterman et al. 2011) using UCS-containing primers, and subsequently cloned into the USER-ATMT-DS vector via the USER technique. The 5' flank-F and Hpt-R primers were used for DNA sequencing to verify correct insertion of the upstream flanking sequences, and the 3' flank-R and Hpt-F primers were used for sequencing to verify correct insertion of the downstream flanking sequences. Upstream and downstream flanking sequences of the three genes were also PCR amplified from V592 genomic DNA using primers containing restriction enzyme sites containing primers. The fragments were subsequently cloned into pKOV21 (Yang et al. 2010) to generate HPT recombination cassettes. HPT recombination cassettes were then PCR amplified from pKOV21 vector, digested by *Bam*HI and *Sal*II, and cloned into pSulPH-EGFP to generate ATMT-PS-SUL vector for each gene.

Primer design and generation of deletion constructs. Replacement of the target gene was validated by PCR. For each gene, primers targeting the upstream (up 5' flank-F) and downstream

Fig. 2. Construction of *VdGARP1* deletion USER *Agrobacterium tumefaciens*-mediated transformation dual-selection binary vector. **A**, Schematic representation of polymerase chain reaction (PCR) primer targeting sites and **B**, verification of *VdGARP1* deletion construct by PCR analyses. The 08781 5' flank-F and Hpt-R primer pair was used to detect the assembly of upstream flanking fragment before HPTBOX, and Hpt-F and 08781 3' flank-R primer pairs were used to detect the assembly of downstream flanking fragment after HPTBOX.

(dn 3' flank-R) of homologous recombination regions were designed for the detection of target gene replacement by HPTBOX. Primer pairs targeting the open reading frame (ORF) of the target gene (ORF-F and ORF-R) were designed to confirm the deletion of target genes. Primer pairs targeting the 5' homologous recombination region (5' flank-F and 5' flank-R) and 3' homologous recombination region (3' flank-F and 3' flank-R) were designed for amplification of homologous recombination regions. Primer pairs (VDAG_02273ORF-F/VDAG_02273ORF-R or VDAG_01995ORF-F/VDAG_01995ORF-R) were used for preparing probes for Southern blot of each genes. Southern blot was performed as described by Gao et al. (2010) and Zhou et al. (2012). All primers are listed in Supplementary Table S1.

RESULTS

Generation of the USER-ATMT-DS binary vector. The traditional multistep cloning method requires subcloning of two homologous sequences at the borders of a PS marker gene (Supplementary Fig. S1A), a time-consuming process. The currently available ATMT binary vector that can be used for single-step cloning does not contain a second negative or positive marker for the elimination of ectopic transformants (Supplementary Fig. S1B); thus, elaborate PCR-based identification of gene-deletion mutants

is required. To further simplify the ATMT-based gene-disruption method, we generated a USER-ATMT-DS binary vector, enabling one-step cloning and elimination of ectopic transformants via *HSVtk* negative selection. The pGKO-Gateway ATMT binary vector (Tian et al. 2011), which contains the *HSVtk* negative-selection marker, was used as a backbone to generate the USER-ATMT-DS binary vector. The unneeded coupled cell division B gene was first replaced with a fragment digested by *HindIII* and *XbaI* from pSulPH-EGFP (Gao et al. 2010), which harbors an *SmaI* cloning site suitable for subsequent introduction of the *HPT* marker gene into the pGKO-Gateway ATMT vector. The resulting intermediate construct was then digested by *HindIII* and *SmaI* and used as vector for subsequent introduction of the *HPT* marker gene and USER cloning sites. The plasmid pRF-HU2 (Frandsen et al. 2008), containing both the *HPT* marker gene fragment (HPTBOX) and the USER cloning sites at both ends, was used as template for PCR amplification, with a forward primer containing a *HindIII* site and a reverse primer containing an *SmaI* site. The resulting PCR product containing an HPTBOX with USER cloning sites at both ends was digested by *HindIII* and *SmaI*, then cloned into the above intermediate vector to generate pGKO-HPT. Thus, PGKO-HPT contains USER cloning sites, the HPTBOX PS marker, and the *HSVtk* negative-selection marker and, therefore, was named USER-ATMT-DS binary vector (Fig. 1).

Fig. 3. Deletion of *VdGAR1* in *Verticillium dahliae* strain V592 using *VdGAR1* USER *Agrobacterium tumefaciens*-mediated transformation dual-selection vector. **A** and **B**, Schematic representations of polymerase chain reaction primer targeting sites and **C**, 08781 up 5' flank-F and 08781 dn 3' flank-R were paired with hygromycin B phosphotransferase (Hpt) primers to detect the replacement of *VdGAR1* gene by HPT. Vd08781ORF-F and Vd08781ORF-R were used to confirm the deletion of *VdGAR1* gene. **D**, Southern blot analysis of genomic DNA isolated from V592 and *vdgarp1* strains, and digested with *HindIII*.

The two USER cloning sites originating from pRF-HU2 were designed for sequential digestion with *PacI* (5'-TTAATTAA-3') and *Nt.BbvCI* to generate four unique 9-bp overhangs, including the 5' overhangs 5'-GGGTTTAAT-3' and 5'-GGACTTAAT-3' that were designed for insertion of upstream (5') flanking sequences, and the 3' overhangs 5'-GGCATTAAT-3' and 5'-GGTCTTAAT-3' that were designed for insertion of downstream (3') flanking sequences. Thus, this one-step USER cloning strategy requires the incorporation of 5'-GGGTTTAAU-3' and 5'-GGACTTAAU-3' sequences into the 5' ends of the respective forward and reverse primers used to amplify the upstream flanking sequences of the target gene. The strategy also requires the incorporation of 5'-GGCATTAAU-3' and 5'-GGTCTTAAU-3' sequences into the 5' ends of the respective forward and reverse primers to amplify the downstream flanking sequences of the target gene. Thus, these four unique overhangs ensure the correct assembly of both the upstream and downstream flanking sequences together with HPTBOX to generate the gene-deletion construct.

Deletion of *VdGARPI* using the USER-ATMT-DS binary vector. In order to test whether the USER-ATMT-DS binary vector is efficient for targeted gene deletion, we first used this vector to generate a *VdGARPI* gene-deletion construct for *V. dahliae* transformation. Primer pairs were designed to amplify the 1-kb upstream and downstream flanking sequences of *VdGARPI*, referring to the genome sequence of *VdLs.17* (Klosterman et al. 2011). Each primer contained the corresponding USER cloning sites at its 5' end for USER enzyme digestion. V592 genomic DNA was extracted and used as a template to amplify both the upstream and downstream flanking fragments. The USER-ATMT-DS vector was sequentially

digested by *PacI* and *Nt.BbvCI* to generate both the vector backbone and HPTBOX. The two fragments were then mixed with the PCR-amplified gene-flanking sequences and incubated with the USER enzyme mix for the indicated time. The resulting product was then transformed into *E. coli*-competent cells to obtain kanamycin-resistant transformants.

PCR amplification using upstream flanking forward (5' flank-F) and HPTBOX reverse (Hpt-R) primer pairs (Fig. 2A) was conducted to confirm correct assembly of the upstream flanking fragment before HPTBOX. HPTBOX forward (Hpt-F) and downstream flanking reverse (3' flank-R) primer pairs were used to confirm correct assembly of the downstream flanking fragment after HPTBOX. In all, 10 of 10 tested transformants were found to be correctly assembled constructs (Fig. 2B), indicating the high efficiency of USER one-step cloning. Correct assembly of the resulting constructs was further confirmed by DNA sequencing. The 5' flank-F and Hpt-R primers were used for sequencing to verify correct insertion of the upstream flanking sequence, and the 3' flank-R and Hpt-F primers were used for sequencing to verify correct insertion of the downstream flanking sequence. A confirmed *VdGARPI* deletion construct was then transformed into *Agrobacterium* strain EHA105 for ATMT of V592. After incubation with *Agrobacterium* carrying the ATMT-*VdGARPI* vector, V592-cultured cells were plated onto PDA plates containing both 5FU and hygromycin B.

Verification of *VdGARPI* deletion in V592. Transformants that grew on PDA containing 5FU and hygromycin B were next examined to assess *VdGARPI* gene replacement. Genomic DNA was isolated from individual transformants. Primers targeting the 5'

Fig. 4. Construction of *VdAG_02273* and *VdAG_01995* gene-deletion USER *Agrobacterium tumefaciens*-mediated transformation dual-selection vectors. **A** and **B**, Schematic representation of PCR primer targeting sites. **C**, Verification of *VdAG_02273* deletion construct by polymerase chain reaction (PCR) analyses; 02273 5' flank-F and Hpt-R primer pairs were used to detect the assembly of upstream flanking fragment before HPTBOX; Hpt-F and 02273 3' flank-R primer pairs were used to detect the assembly of downstream flanking fragment after HPTBOX. **D**, Verification of *VdAG_01995* deletion construct by PCR analyses. The 01995 5' flank-F and Hpt-R primer pairs were used to detect the assembly of upstream flanking region 5' of HPTBOX. Hpt-F and 01995 3' flank-R primer pairs were used to detect correct assembly of downstream (3') flanking region after HPTBOX.

upstream flanking sequence (up 5' flank-F) and the 3' downstream flanking sequence (dn 3' flank-R) of *VdGARP1* were designed and paired with Hpt primers to determine whether the target gene was successfully replaced by the *HPT* gene (Fig. 3A). Primer pairs targeting the *VdGARP1* coding sequence (*VdGARP1*-F and *VdGARP1*-R) were used to confirm deletion of the *VdGARP1* gene (Fig. 3B). The PCR

analyses indicated that 5 of 5 HPT-resistant transformants were *VdGARP1* gene knock-out mutants (Fig. 3C).

Deletion of the *VdGARP1* gene in the *V592* genome was further confirmed by Southern blot analysis. Genomic DNA samples isolated from the *V592* wild-type (WT) strain and one HPT-resistant transformant (named *vdgarp1*) were digested by *Hind*III restriction

Fig. 5. Deletion of *VDAG_02273* and *VDAG_01995* in *V592* using USER *Agrobacterium tumefaciens*-mediated transformation dual-selection (ATMT-DS) vectors. **A**, Transformants grown on potato dextrose agar (PDA) plates with or without 5-fluoro-2'-deoxyuridine (5FU). *Verticillium* culture incubated with agrobacteria carrying ATMT-*VDAG_02273* vector was plated onto plates as indicated. **B**, Verification of *VDAG_02273* deletion in *V592*; 02273 up 5' flank-F and 02273 dn 3' flank-R were paired with hygromycin B phosphotransferase (Hpt) primers to detect the replacement of *VDAG_02273* gene by HPT. Primers 02273ORF-F and 02273ORF-R were used to confirm the deletion of *VDAG_02273* gene. **C**, Verification of *VDAG_01995* deletion in *V592*. The 01995 up 5' flank-F and 01995 dn 3' flank-R primers were paired with Hpt primers to detect the replacement of *VDAG_01995* gene by HPT. Primers 01995ORF-F and 01995ORF-R were used to confirm the deletion of *VDAG_01995* gene. Genomic DNA samples isolated from **D**, *V592* and *vd02273* or **E**, *V592* and *vd01995* were digested by *Hind*III and subjected to Southern blot analysis.

enzyme. A 900-bp probe targeting the *VdGARP1* gene was able to detect the presence of the *VdGARP1* coding sequence in the V592 genome (Fig. 3D). However, the same probe was unable to detect the presence of the *VdGARP1* gene in the *vdgarp1* mutant, indicating loss of the genomic *VdGARP1* fragment (Fig. 3D).

The USER-ATMT-DS vector is efficient for gene deletion in *V. dahliae*. In order to test whether the USER-ATMT-DS method is generally applicable for gene deletion in *V. dahliae*, the vector was used to construct other vectors for deletion of an additional two genes, *VDH1* (VDAG_02273) (Klimes and Dobinson 2006) and *VdNLP2* (VDAG_01995) (Zhou et al. 2012) in strain V592. Primer pairs were designed to amplify approximately 800 bp of flanking sequences of the above two genes, referring to the genomic sequence of strain VdLs.17 (Klosterman et al. 2011). Upstream flanking forward and reverse primer pairs and downstream flanking forward and reverse primer pairs (Fig. 4A and B) were designed to identify correctly assembled constructs for VDAG_02273 (Fig. 4C) and VDAG_01995 (Fig. 4D). The deletion constructs were verified by DNA sequencing and subsequently used for ATMT.

In the USER-ATMT-DS vector method, most of the ectopic transformants are expected to be killed by 5FU due to the presence of the *HSVtk* gene. To examine the efficiency of *HSVtk* negative selection, *Verticillium* cultures were incubated with *Agrobacterium* spp. carrying the ATMT-VDAG_02273 vector and plated onto PDA plates with or without 5FU. The addition of 5FU significantly reduced the number of transformants (Fig. 5A), indicating the elimination of ectopic transformants. The transformants that grew on PDA containing 5FU and hygromycin B were further verified for target gene deletion by PCR using primer pairs targeting the VDAG_02273 (Fig. 5B) and VDAG_01995 (Fig. 5C) coding sequences. Replacement of the target gene by HPT was verified by pairing primers targeting the upstream and downstream sequences of homologous recombination regions with Hpt primers (Fig. 5B and C). Gene deletion was further confirmed in one of the PCR-verified VDAG_02273 transformants (named *vd02273*) and one of the PCR-verified VDAG_01995 transformants (named *vd01995*) by Southern blot analysis. Genomic DNA samples isolated from the WT V592 strain as well as the *vd02273* and *vd01995* strains were digested by *HindIII* restriction enzyme and subjected to Southern blot. The probe targeting the VDAG_02273 gene was able to detect the presence of the VDAG_02273 coding sequence in the WT V592 genome but not in the *vd02273* mutant (Fig. 5D). Similarly, the probe targeting the VDAG_01995 gene successfully detected the presence of the VDAG_01995 coding sequence in the WT V592 genome but failed to detect its presence in the *vd02273* mutant (Fig. 5E).

We previously used the ATMT-PS-SUL binary vector to generate gene-deletion mutants in the V592 strain. The ATMT-PS-SUL binary vector carries a second PS marker gene, *sulfonylurea* (*SUL*), which was bordered by two homologous recombination regions, in addition to the *HPT* marker gene to identify correct gene-replacement mutants (Supplementary Fig. S2A). In this method, mutants with successful target gene replacement carry only the *HPT* resistance gene, whereas ectopic transformants carry both *HPT* and *SUL* resistance genes. *HPT*

is the first PS marker used to identify all transformants, including both gene-replacement mutants and ectopic transformants. A second *SUL* selection step, which is achieved by replica plating HPT-resistant transformants onto a plate containing both HPT and *SUL*, is required for the identification of correct homologous recombinants. To compare the efficiency of gene-deletion mutant generation achieved with the different methods, we also generated *VdGARP1*, *VDAG_02273*, and *VDAG_01995* gene-deletion constructs using the ATMT-PS-SUL binary vector, and transformed the V592 strain with these vectors to knock out the corresponding genes. ATMT-PS-SUL transformants subjected only to the initial step of HPT selection were used to evaluate the efficiency of gene-deletion mutant generation using only the HPT PS marker. ATMT-PS-SUL transformants subjected to both HPT and *SUL* selection were used to evaluate the efficiency of gene-deletion mutant generation using the two PS markers. Meanwhile, USER-ATMT-DS transformants subjected to both HPT and 5FU selection were used to evaluate the efficiency of gene-deletion mutant generation using both the positive and negative-selection markers. PCR amplification using primer pairs targeting the corresponding coding sequences in the genome was used for verification of the correct gene-replacement mutants. The results indicated that single positive selection (e.g., HPT) has a relatively lower efficiency (approximately 20%) (Table 1) compared with the other two methods. Although the ATMT-PS-SUL method, which employs two PS markers, has high efficiency (approximately 80%) comparable with that of the USER-ATMT-DS method (approximately 80%), it requires an additional replica-plating step (Table 1). By contrast, the USER-ATMT-DS-based strategy does not require the replica-plating step and retains high selection efficiency.

DISCUSSION

The efficiency of ATMT-based gene disruption in fungal species largely lies in the generation of a gene-deletion construct and the selection of gene-replacement recombinants. Several single-step cloning strategies, such as In-Fusion cloning (Frandsen et al. 2008; Zhu et al. 2007), the OSCAR method (Paz et al. 2011), and the USER cloning technique (Frandsen et al. 2008, 2011; Santhanam and Thomma 2013), have been introduced into the ATMT-PS binary vector to speed up the construction procedure. Compared with the In-Fusion cloning strategy, both the OSCAR method and the USER cloning strategies offer relatively higher efficiency for precise assembly of multiple fragments. The OSCAR method was used for rapid and robust generation of VDAG_02161 and VDAG_09930 gene-deletion constructs, and it was further verified to be efficient for deletion of these genes in *V. dahliae* (Paz et al. 2011). The USER-ATMT vector has been successfully used for the generation of a *VdSge1* gene-deletion construct (Santhanam and Thomma 2013), which regulates the expression of several secreted protein genes in *V. dahliae*. However, both OSCAR-ATMT and USER-ATMT vectors contain only the HPT selection marker; thus, elaborate methods are needed for the identification of fungal gene-replacement recombinants.

TABLE 1. Efficiency (%) for generation of gene-deletion mutants of *Verticillium dahliae* by different methods

V592 gene ID	Percent gene-deletion efficiency (correct deletion mutants/tested transformants) ^a		
	USER-ATMT-DS	ATMT-PS-SUL	
	PDA/5FU + hygromycin B	PDA/hygromycin B	PDA/SUL and PDA/hygromycin B
<i>VdGARP1</i>	80 (16/20)	20 (4/20)	77 (10/13)
<i>VDAG_02273</i>	85 (17/20)	30 (6/20)	75 (12/16)
<i>VDAG_01995</i>	70 (14/20)	15 (3/20)	79 (11/14)
Average efficiency	78	22	77

^a In all, 13 to 20 transformants generated by different methods, as indicated, were tested by polymerase chain reaction to verify the deletion efficiency of target genes in V592 genome. ATMT = *Agrobacterium tumefaciens*-mediated transformation, DS = dual selection, PS = positive selection, SUL = sulfonylurea, PDA = potato dextrose agar, and 5FU = 5-fluoro-2'-deoxyuridine. Numbers in parentheses = ratio of the number of correct deletion mutants to that number of tested transformants, which indicates efficiency for generation of gene-deletion mutants.

The SUL PS marker in the ATMT-PS-SUL vector simplifies the procedure for characterization of fungal gene-replacement mutants. This method requires replica plating of numerous fungal transformants onto PDA plus hygromycin B plates as well as PDA plus SUL plus hygromycin B plates; thus, it is still time consuming and labor intensive. The introduction of a second negative-selection marker is an efficient method for facilitating this procedure. *HSVtk* negative selection enables elimination of ectopic transformants generated via nonhomologous recombination and, thus, can be used to facilitate the identification of targeted gene-replacement mutants. This approach was deemed highly efficient for gene deletion in *M. grisea* and *F. oxysporum* (Khang et al. 2005). In *V. dahliae*, the ATMT-DS vector has also been used for the disruption of several genes involved in fungal pathogenesis and development. For example, the pGKO2 binary vector (Khang et al. 2005) carrying the *HSVtk* negative-selection marker has been used for deletion of *VdPKAC1* (Tzima et al. 2010), *VdSNF1* (Tzima et al. 2011), and *VGB* (Tzima et al. 2012). This vector allows high-efficiency identification of gene-replacement recombinants but requires multiple cloning steps for the generation of gene-deletion construct.

Recent progress in genome sequencing and assembly of *V. dahliae* (Klosterman et al. 2011) is anticipated to facilitate large-scale gene-disruption experiments aimed at analyzing this representative soilborne pathogen. However, an ATMT vector is lacking for simplified cloning and efficient negative selection for the purpose of high-throughput gene deletion. The USER-ATMT-DS vector that we constructed contains both the USER cloning sites and the *HSVtk* negative-selection marker. The applicability and high efficiency of this vector were validated through deletion of three different genes in the same *V. dahliae* strain. The data presented herein provide strong evidence that the USER-ATMT-DS vector allows for both convenient single-step cloning and highly efficient selection of targeted gene-replacement mutants; thus, it may be useful for large-scale gene disruption in *Verticillium* spp. Because the *HSVtk* negative-selection marker is also applicable in other fungal pathogens such as *M. grisea* and *F. oxysporum* (Khang et al. 2005), the USER-ATMT-DS vector is likely applicable for use in efficient gene disruption in these fungal species.

ACKNOWLEDGMENTS

We thank Y.-L. Peng for providing pKOV21 vector, X.-F. Dai for providing pGKO-Gateway vector, and R. J. N. Frandsen for providing pRF-HU2 vector. This work was supported by grants from the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB11020600) and the China Transgenic Research and Commercialization Key Special Project (2014ZX00800908B).

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