the plant journal

SEB Society for Experimental Biology

The Plant Journal (2016) **86,** 89–101 doi: 10.1111/tpj.13148

TECHNICAL ADVANCE

Spike-dip transformation of *Setaria viridis*

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Received 16 November 2015; revised 9 February 2016; accepted 15 February 2016; published online 1 March 2016.
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SUMMARY

Traditional method of *Agrobacterium*-mediated transformation through the generation of tissue culture had limited success for *Setaria viridis,* an emerging C4 monocot model. Here we present an efficient *in planta* method for *Agrobacterium*-mediated genetic transformation of *S. viridis* using spike dip. Pre-anthesis developing spikes were dipped into a solution of *Agrobacterium tumefaciens* strain AGL1 harboring the β-glucuronidase (GUS) reporter gene driven by the cauliflower mosaic virus 35S (CaMV35S) promoter to standardize and optimize conditions for transient as well as stable transformations. A transformation efficiency of 0.8 ± 0.1% was obtained after dipping of 5-day-old S3 spikes for 20 min in *Agrobacterium* cultures containing *S. viridis* spike-dip medium supplemented with 0.025% Silwet L-77 and 200 μM acetosyringone. Reproducibility of this method was demonstrated by generating stable transgenic lines expressing β-glucuronidase plus (GUSplus), green fluorescent protein (GFP) and *Discosoma* sp. red fluorescent protein (DsRed) reporter genes driven by either CaMV35S or intron-interrupted maize ubiquitin (Ubi) promoters from three *S. viridis* genotypes. Expression of these reporter genes in transient assays as well as in T1 stable transformed plants was monitored using histochemical, fluorometric GUS activity and fluorescence microscopy. Molecular analysis of transgenic lines revealed stable integration of transgenes into the genome, and inherited transgenes expressed in the subsequent generations. This approach provides opportunities for the high-throughput transformation and potentially facilitates translational research in a monocot model plant.

Keywords: *Agrobacterium*-mediated spike dip, *in planta*, monocots, *Setaria viridis*, transient and stable transformation, technical advance.

INTRODUCTION

Genetic transformation is a 'hallmark' technique for translational research in transgenic plants. Out of several genetic transformation methods available, *Agrobacterium*-mediated genetic transformation is the preferred choice for introducing genes of interest into target plant species (Gelvin, 2003). This gene transfer method possess several advantages over others; a relative high transformation efficiency, integration of large segments of transfer DNA (T-DNA) into the host genome, low copy number of transgene, stable inheritance, fewer rearrangements of T-DNA with less transgene silencing in subsequent generations (Birch, 1997; Gelvin, 2003). The conventional method of *Agrobacterium*-mediated genetic transformation of relatively recalcitrant monocots involves labor-intensive and time-consuming *in vitro* tissue cultures where transformed undifferentiated callus tissue becomes organogenic to regenerate whole T0 plants by highly skilled personnel (Birch, 1997; Sood *et al.*, 2011; Hiei *et al.*, 2014). These *in vitro* tissue culture phases are usually genotype dependent and frequently result in somaclonal variations (Cheng *et al.*, 2004; Wang and Wang, 2012). In addition, the production of genetic chimeras with morphological abnormalities and reduced fertility including significant epigenetic changes occur (Wang and Wang, 2012). It is therefore highly desirable to develop an *Agrobacterium*-mediated transformation procedure for monocot plants that obviates these obstacles by eliminating the tissue culture and regeneration phases, and also accelerating the time needed for the generation of the transgenic plants.

A simple and rapid *Agrobacterium*-mediated transformation using *in planta* floral dip in *Arabidopsis thaliana*, a dicot model species, is in common use (Clough and Bent,

1998). This routine transformation system for Arabidopsis has contributed significantly to the knowledge obtained from this model plant (Bent, 2000). A similar floral transformation approach was developed for few dicot plant species (Curtis and Nam, 2001; Yasmeen *et al.*, 2009; Liu *et al.*, 2012; Bastaki and Cullis, 2014) including the model legume *Medicago truncatula* (Trieu *et al.*, 2000), although not reproducible.

Several parameters like: (i) developmental growth stage of target tissue or explant; (ii) genetic background of the *Agrobacterium* strains; (iii) composition of culture medium and surfactants; and (iv) cell density and co-cultivation time, significantly influence the efficiency of *Agrobacterium*-mediated transient and stable transformations in dicots (Cheng *et al.*, 2004; Kim *et al.*, 2009; Ghedira *et al.*, 2013). The young immature flowers of Arabidopsis (Clough and Bent, 1998) and the pre-anthesis spikes of wheat at the early or mid uni-nucleate microspore stage (Zale *et al.*, 2009) are the most susceptible target tissues amenable to *Agrobacterium*. In addition, depending on the target plant species some *Agrobacterium* strains are more virulent than others (Ghedira *et al.*, 2013). The GV3101 strain (Koncz and Schell, 1986) is one of the commonly used laboratory strains useful for several dicot transformations, whereas the AGL1 strain (Lazo *et al.*, 1991), is the most widely used *Agrobacterium* strain in monocot transformations (Wu *et al.*, 2008; Alves *et al.*, 2009; Zale *et al.*, 2009). Applications of several surfactants like Tween 20, Triton X-100 and Silwet L-77 to improve efficiency of the *Agrobacterium*-mediated transformations have been reported for many plant species (Clough and Bent, 1998; Kim *et al.*, 2009). The culture medium composition, cell density and co-cultivation time are also critical factors for successful *Agrobacterium*-mediated transient and stable transformations (Clough and Bent, 1998; Kim *et al.*, 2009; Chen *et al.*, 2010).

The *Setaria viridis* with its small diploid genome (approximately 510 Mb) (Bennetzen *et al.*, 2012), little size, simple growth requirements, short life cycle, C4 photosynthesis and is a close relative of major food (maize and sorghum) and biofuel (switchgrass) monocots of the Panicoideae subfamily represents an ideal emerging monocot model (Brutnell *et al.*, 2010, 2015; Li and Brutnell, 2011). Several resources, for example, genome sequencing (Bennetzen *et al.*, 2012); a method for performing crosses (Jiang *et al.*, 2013); seed germination (Sebastian *et al.*, 2014); feedstock characterization (Petti *et al.*, 2013); molecular diversity of populations (Jia *et al.*, 2013); and genomic (Huang *et al.*, 2014) and transcriptomic (Xu *et al.*, 2013; John *et al.*, 2014) data sets including a traditional way of *Agrobacterium*-mediated transformation through intervention of tissue culture (Brutnell *et al.*, 2010), have been developed or underway for this model plant. Recently, the feasibility of the floral-dip transformation of *S. viridis* was suggested (Martins *et al.*, 2015), unfortunately the method was not optimized, the transformation efficiency was not determined and the method reproducibility was not established. Although, an analogous method has been applied to transform a few large and polyploid genome monocot species, they had limited success (Zale *et al.*, 2009; Mu *et al.*, 2012). Therefore, due to the lack of a well established *in planta Agrobacterium*-mediated transformation protocol for any small diploid genome monocot model, establishing a similar transformation protocol of *S. viridis* would enable significant progress in the field.

Here we report the development and optimization of conditions for *in planta Agrobacterium*-mediated genetic transformation of *S. viridis* using spike-dip method. Molecular analysis of transgenic lines showed stable integration of transgenes into the genome, and inherited transgenes expressed in the subsequent generations. The reproducibility of this method was demonstrated by generating stable transgenic lines from three *S. viridis* genotypes using five reporter gene constructs that produced fertile transgenic plants within 8–10 weeks' time. This protocol provides an *in planta* monocot transformation system, and will be widely applicable to study gene function and gene silencing.

RESULTS

Selection of an appropriate medium for spike-dip transformation

To optimize different parameters for optimal transformation, we initially used an *A. tumefaciens* strain AGL1 harboring a 35S::GUS (pCAMBIA1201) reporter gene construct (Figure S1a) to transform *S. viridis* spikes. The pCAMBIA1201 vector includes a β-glucuronidase (GUS) reporter gene from *Escherichia coli* with an intron from the castor bean catalase gene within the coding sequence to ensure that expression of glucuronidase activity was derived from plant cells, not from expression by residual *A. tumefaciens* cells. Transient transformation was measured by monitoring the expression of GUS using fluorometric GUS assays and/or histochemical GUS activity after 3 days post dipped (DPD), while the efficiency of stable transformations was calculated by germinating T1 seeds on plates containing hygromycin. Five media constituents, *S. viridis* infiltration (Svl), *S. viridis* spike dip (SvSD), Murashige and Skoog (MS), sucrose (5%) and a combination of MS + sucrose, previously shown to be useful for plant transformations were tested. Relatively low transformation rates of both transient and stable transformations were found in Svl and MS media (Figure 1a). Sucrose (5%) and addition of sucrose to MS medium increased two to five-fold the transformation rates as compared with MS alone, suggesting that simple media had no effect on transformation efficiencies. The complex SvSD medium showed significantly higher transient as well as stable [0.7% ± 0.1 mean

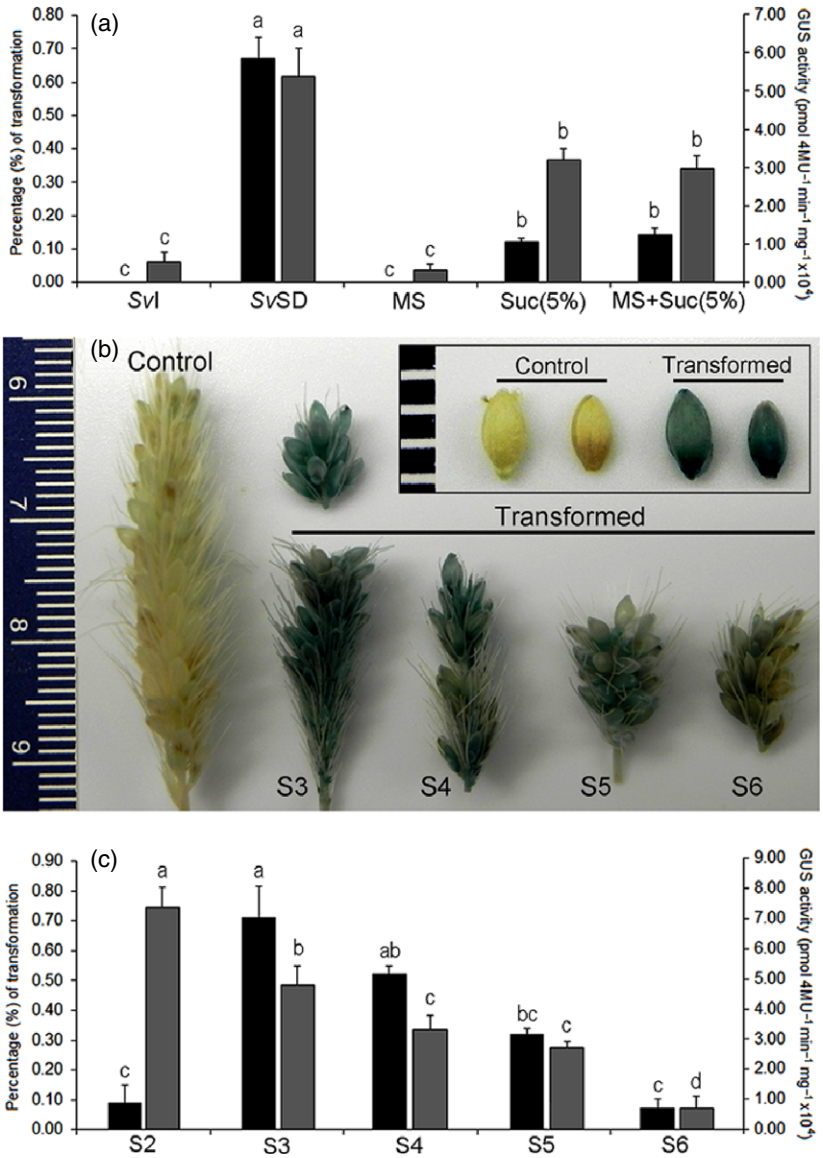

Figure 1. Optimized conditions for spike-dip transformation.
(a) Effect of media composition on transformation efficiency.
(b) Histochemical GUS assay of untransformed, mock transformed control, transformed spikes expressing GUS at different developmental stages (S3 to S6). The inset panel shows an enlarged view of untransformed and transformed florets after GUS assays.
(c) Spike development stages (S1 to S6) most amenable to high transformation efficiency.
The *A. tumefaciens* strain AGL1 harboring 35S:: GUS (pCAMBIA1201) reporter gene construct was used for these experiments. Histochemical staining and fluorometric GUS assays were conducted after 3 DPD. SvI, *S. viridis* infiltration; SvSD, *S. viridis* spike dip; MS, Murashige and Skoog; Suc (5%), sucrose (5% w/v); and MS + Suc (5%), MS medium supplemented with 5% sucrose. Efficiency of stable transformation was calculated by germinating T1 seeds on hygromycin plates.
Data are the mean ± SD (n = 3). ^{a,b,c}Different letters above the bars indicate significant differences at the $P \leq 0.05$ level as tested by Tukey–Kramer HSD. Primary and secondary y-axes represent stable (% of transformation) and transient (fluorometric GUS assays) transformations, respectively. See Experimental procedures and Figures S1–4 for more details.

transformation rate ± standard deviation (SD)] transformation among all five different combinations of media tested (Figure 1a). Glycerol and sucrose were found to be critical components of the SvSD medium and elimination of either component from the SvSD medium resulted in no transformation event. The pH of the medium was adjusted to 5.8 and the surfactant Silwet L-77 (0.025%) was used. The addition of BAP (10 mg L^{-1}) to SvSD had no effect on spike-dip transformation of *S. viridis*, therefore SvSD alone was used in subsequent experiments.

Identification of the optimal foxtail spike developmental stage for *Agrobacterium transformation*

To identify the ideal *S. viridis* spikes growth stage amenable to *Agrobacterium* transformation, we categorized the spike development process from pre-anthesis to post-anthesis into seven distinct successive stages (S1 to S7) based on the first visualization of its emergence among leaf sheath, auricle and flag leaf (Figure S2). The spike lengths and the time required for each developmental stage of *S. viridis* accession A10.1 is given in Figure S2. At the early booting phase, primary tillers bearing spikes needed an average of 1.8 ± 0.8 days to reach a length of 3.7 ± 0.3 cm at S1, while it reached to maximum size of 5.9 ± 0.30 cm in 7.4 ± 1.3 days at S4 (Figure S2). We found that pre-anthesis spikes at S1 to S5 stages were susceptible to transformation, whereas early (S6) or late (S7) post-anthesis spikes were recalcitrant (Figures 1b,c and S2). We also observed that spikes of 5.5 ± 0.3 cm size after 5.4 ± 0.8 days of emergence at S3 were the most

amenable to *Agrobacterium* infection in both transient and stable transformations. Histochemical staining and fluorometric GUS assays revealed that, as the spikes approached anthesis, the susceptibility of spikes to *Agrobacterium* transformation decreased from S2 to S6 (Figure 1b,c).

Suitable dipping time and the effect of surfactants

The minimum dipping time required for the highest transformation efficiency was determined. *S. viridis* transient transformation did not change when the spikes were dipped between 10 and 30 min (Figure 2a). For stable transformations, the highest efficiency was attained with 20 min of dipping, whereas the transformation efficiency decreased at 40 min (Figure 2a). Levels of transient expression was higher in the presence of 0.01% Silwet L-77, while highest transformation was obtained in the presence of 0.025% Silwet L-77 and both transient and stable transformations decreased in the presence of amounts of surfactants higher than 0.1% (Figure 2b).

Transformation efficiency of *Agrobacterium* strains

The efficiency of four *A. tumefaciens* strains (AGL1, EHA105, GV3101 and LBA4404) in *S. viridis* transformation was evaluated by measuring GUS activity (Figure 2c). All the *Agrobacterium* strains were equally effective in transforming *S. viridis* transiently (Figure 2c). Nevertheless, for stable transformation the order of efficiency was EHA105 = AGL1 > LBA4404 = GV3101 (Figure 2c). We assessed the effect of the *Agrobacterium* growth phase on transformation efficiency using the strain EHA105 and four growth phases representing lag phase (OD_{600} = 0.2), early log phase (OD_{600} = 0.5), late log phase (OD_{600} = 1.0), and stationary phase (OD_{600} = 1.5) (Figure 2d). Low transformation was attained at the lag phase, higher rates were seen at the log phases and significant lower rates were obtained at the stationary phase (Figure 2d). *Agrobacterium* at the stationary phase induced severe spike yellowing, wilting and bleaching. Although, *Agrobacterium* at early or late log phases were equally effective in transiently transform, the late log phase was more efficient than the early log phase for stable transformation of *S. viridis* (Figure 2d).

Validation of spike-dip transformation and inheritance of marker genes in the T1 generation

To validate the optimized protocol established with the *A. tumefaciens* strain AGL1 harboring the 35S::GUS binary vector (Figure S1a), we transformed *S. viridis* using EHA105 harboring four more constructs, namely, 35S::GUSplus, Ubi::GUSplus, 35S::GFP and Ubi::DsRed (Figure S1b–e). The GUSplus gene was originally isolated from a *Staphylococcus* species and is more stable at higher temperatures and in fixatives than the GUS gene. The *Aequoria victoria* green fluorescent protein (GFP) and the *Discosoma* sp. red fluorescent protein (DsRed) reporter

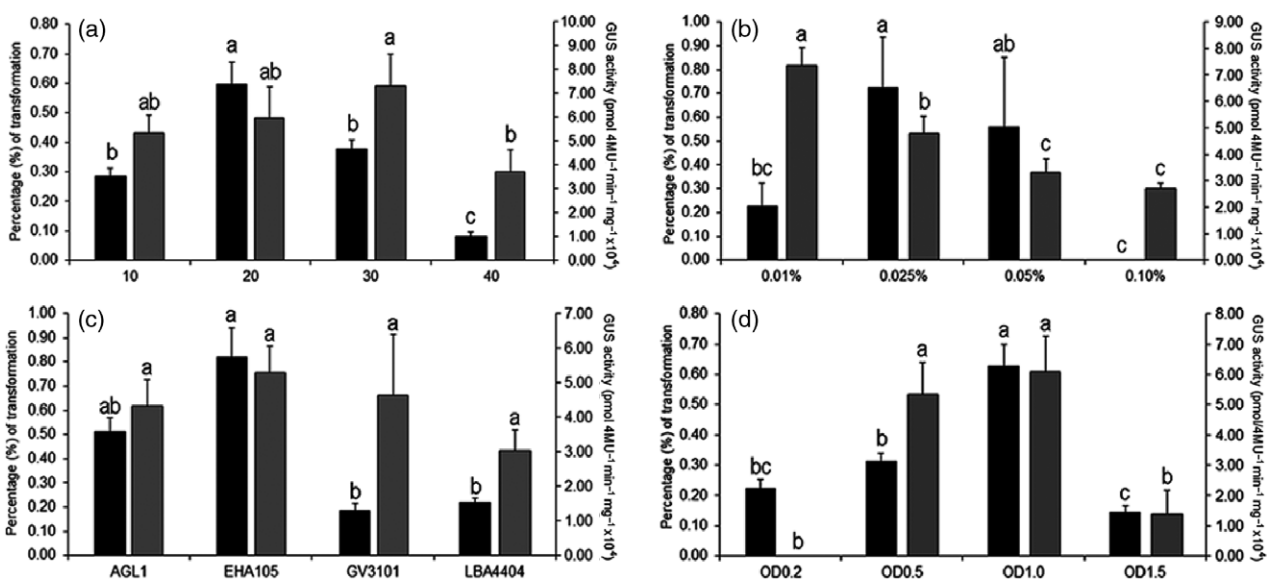

Figure 2. Standardization of spike-dip transformation.
(a) Minimum time requirement for highest transformation efficiency.
(b) Effect of Silwet-L77 concentration on stable and transient transformation.
(c) Efficiency of *Agrobacterium* strains in spike-dip transformation.
(d) Growth stage and concentration of *A. tumefaciens* strain EHA105, harboring 35S::GUS (pCAMBIA1201) reporter gene construct that was used for figure (a, b, d) experiments. Transformation efficiency and transient transformation were conducted as described in Figure 1 (see Experimental procedures for more detail). Data are the mean ± SD (n = 3). [a,b,c]Different letters on the bars indicate significant differences at the $P \leq 0.05$ level as tested by Tukey–Kramer HSD test. Primary and secondary y-axes represent stable (% of transformation) and transient (fluorometric GUS assays) transformations, respectively.

genes were under the control of CaMV35S and Ubi promoters, respectively. In addition, the transformation with these vectors allowed us to examine the efficacy and strength of the CaMV35S and Ubi promoters for *in planta* transformation of *S. viridis*. One day before dipping, plants with single primary tiller bearing a spike at S3 were identified. The spikes were gently unsheathed and labelled for appropriate construct (Figure S3a) and the remaining secondary spikes were clipped off from the plants. *Agrobacterium* cultures containing the plasmids were prepared a day before dipping by inoculating 500 μl of glycerol stocks into 50 ml of YEB medium. The following day, the *Agrobacterium* culture density was adjusted to OD_{600} = 1.0 with SvSD medium after addition of 200 μM acetosyringone. Following pre-induction for 1 h, the spikes were immersed into the *Agrobacterium* culture for 20 min with occasional gentle agitation (Figure S3b), before placing them under a plastic dome for 24 h (to retain humidity), either in a growth chamber or in a culture room under the low light intensity. DsRed was highly expressed under the control of the Ubi promoter (Figure 3c) as compared with GFP driven by CaMV35S (Figure 3d). Fluorometric measurements also showed four to six-fold higher GUS activity from GUSplus lines driven by Ubi promoter as compared with lines driven by CaMV35S (Figure 3e). As the transformed spikes approached anthesis, the plants were placed inside arecones to prevent cross pollination and were kept under normal greenhouse conditions for seed setting (Figure S3c). Mature T1 seeds were harvested and screened for stable transformation events (Figure S4). The expression and inheritance of the marker genes in the T1 generation was monitored (Figure 4). We observed expression of GUS and GUSplus reporter genes in mature T1 seeds, T1 seedlings of 3 and 5 days post germination (DPG) old, and mature leaves of T1 plants (Figure 4a–d). In addition, GFP and DsRed fluorescence was also seen in stable transformed T1 lines (Figure 4e–h).

Selection of putative transformants and transformation efficiency

A range of transformation efficiency of 0.5–0.7% was obtained using *Agrobacterium* strain AGL1 expressing 35S::GUS (Figures 1a, 2c and Figure S1a). For further validation, we tested several transgenic lines generated from all reporter gene constructs using *Agrobacterium* strain EHA105 and obtained a range of 0.5–0.8% efficiency of stable transformation with the genotype A10.1 (PI 66942/Ames 31045), based on the selection of T1 seeds on hygromycin plates (Figure 2c and Table 1). The germination of false positives during the selection of T1 seeds on hygromycin-containing plates was observed after 7 DPG (Figure S4c–d). These seedlings grew slowly and turned yellow to brown and eventually died. On the other hand, the hygromycin-tolerant transformed seedlings remained

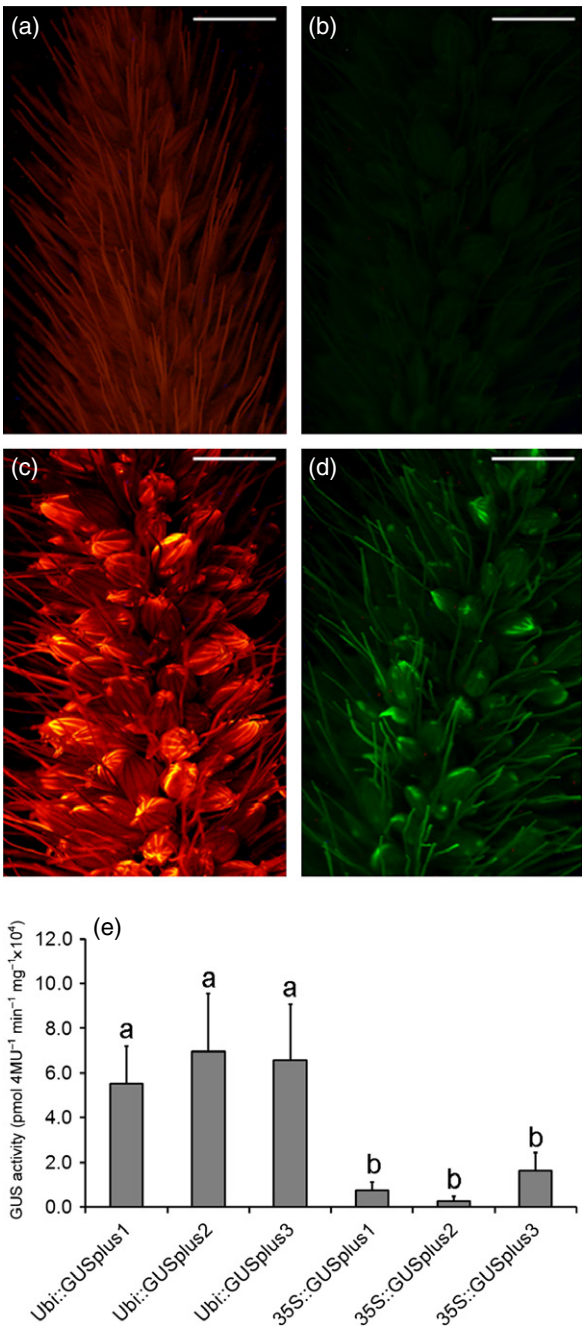

Figure 3. Expression of fluorescent reporter genes in transient transformation assays.
(a, b) Untransformed control and mock (pCAMBIA1201) transformed spikes measured through DsRed and GFP filters.
(c, d) Transient expression of DsRed and GFP reporter genes from spikes transformed with Ubi::DsRed (pGWB17-UbiDsRED-UbiBAR) and 35S::GFP (pH7m24GW35Sp-GFP) constructs, respectively. Pictures were taken using a Leica MZFLIII fluorescence stereomicroscope coupled with respective filters and CCD camera after 5 DPD. Scale = 1 mm. See Experimental procedures and Figure S1 for further detail.
(e) Transient fluorometric measurement of GUS expression from *S. viridis* spikes transformed with Ubi::GUSplus and 35S::GUSplus reporter gene constructs after 3 DPD. Data are the mean ± SD (n = 3). ^{a,b}Different letters on the bars indicate significant differences at the $P \leq 0.05$ level as tested by the Tukey–Kramer HSD test.

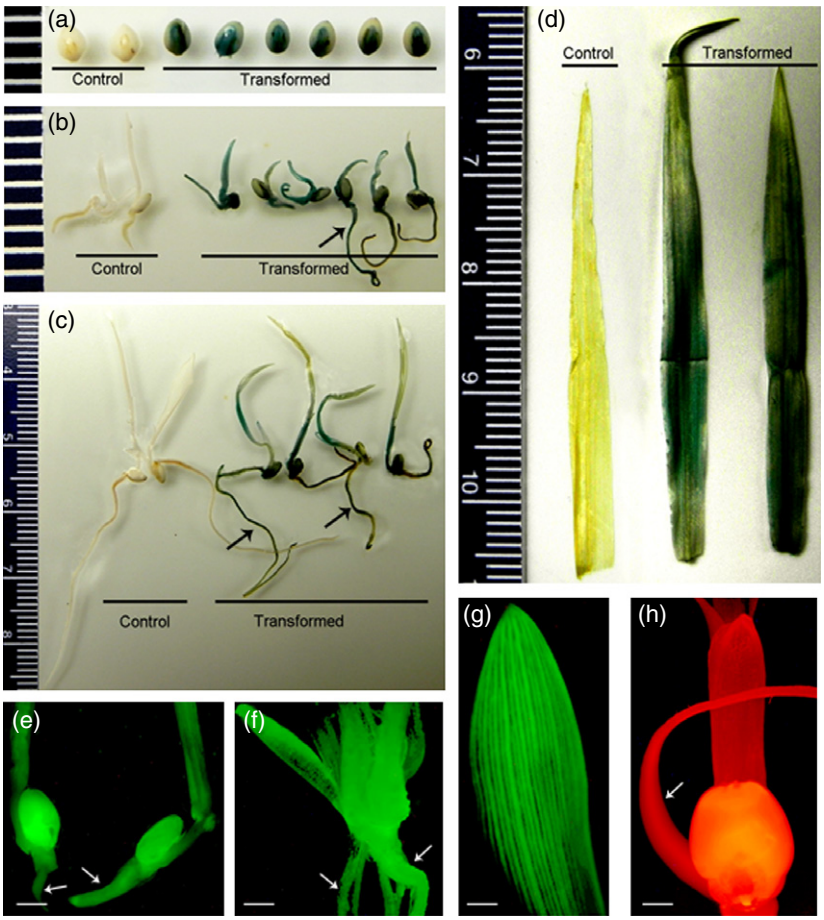

Figure 4. Stable expression of reporter genes in the T1 generation.
(a) Histochemical GUS assay of mature seeds of untransformed or mock transformed controls and T1 lines transformed with the 35S::GUS (pCAMBIA1201) reporter gene.
(b, c) Histochemical assay for GUSplus reporter gene activity in 3 DPG and 5 DPG T1 seedling transformed with the Ubi::GUSplus (pH7m24GW pUbi-GUSplus) and the 35S::GUSplus (pH7m24GWp35S-GUSplus) constructs, respectively.
(d) Histochemical GUS assay of mature leaves from untransformed control plant and T1 plants transformed with the 35S::GUS (pCAMBIA1201) construct.
(e–g) Expression of the GFP reporter gene in three DPG seedling, seven DPG plantlets and 10 DPG leaf of T1 lines transformed with the 35S::GFP (pH7m24GW35Sp-GFP) construct.
(h) Expression of the DsRed reporter gene in five DPG T1 seedling transformed with the Ubi::DsRed (pGWB17-UbiDsRED-UbiBAR) construct. Histochemical GUS staining photos were captured using a Nikon COOLPIX P530 digital camera while fluorescence pictures were taken using a Leica MZFLIII fluorescence stereomicroscope coupled with respective filters and a CCD camera. Arrows indicate roots. Scale bar represents 1 mm.

green with well developed roots (Figure S5). When Ubi::DsRed T1 seeds were selected on plates containing both hygromycin and bialaphos, the transformation efficiency was 0.5% and no false positives were developed (Table 1). To further evaluate the reproducibility of this protocol, two other genotypes, 132 (PI Ames 28193) and 98HT-80 (PI 649320) were transformed using *A. tumefaciens* strain EHA105 containing 35S::GUS construct and obtained 0.7 and 0.8% of stable transformation rates, respectively (Table 1).

Molecular analysis of transgenic plants

To assess the stable integration of transgenes in the *S. viridis* genome, we used polymerase chain reaction (PCR) and gene-specific primer pairs (Table S1) to test the presence of the reporter GUS, GUSplus, GFP and DsRed, and the selectable marker HptII genes (Figure 5). Agarose gel electrophoresis analysis of the PCR products obtained from randomly selected independent transgenic plants revealed the presence of the respective size bands, specific for each reporter gene and selectable marker (Figure 5a,b). No amplification was found in the PCR products of untransformed control (UT-C) plant DNA under the identical conditions. Amplification of a native endogenous tubulin (*TUB*) gene from genomic DNA of *S. viridis* further validates our experiment (Figure 5c).

We confirmed transgene integration and determine the copy number of the integrated T-DNA in the genome by

Table 1 Efficiency of spike-dip transformation by *Agrobacterium tumefaciens* strain EHA105 harboring different reporter gene constructs

Constructs/lines	Genotype	Number of seeds tested	Selection (mg L^{-1})	Seeds germinated	Percentage (%) of transformation ± SD
UT-C (WT)	A10.1	252	No	245 (3)[b]	ND
UT-C (WT)	A10.1	120	Hyg 30	0 (2)	ND
35S::GUS	A10.1	567 (4)[a]	Hyg 30	4 (12)	0.7 ± 0.2
35S::GUSplus	A10.1	316 (1)	Hyg 30	2 (6)	0.6 ± 0.0
Ubi::GUSplus	A10.1	176 (1)	Hyg 30	1 (3)	0.6 ± 0.0
35S::GFP	A10.1	606 (4)	Hyg 30	4 (10)	0.6 ± 0.1
Ubi::DsRed	A10.1	413 (3)	Hyg 30 + Bia 3	2 (0)	0.5 ± 0.2
35S::GUS	132	289 (2)	Hyg 30	2 (4)	0.7 ± 0.0
35S::GUS	98HT-80	372 (3)	Hyg 30	3 (7)	0.8 ± 0.1

[a]Number of spike dip experiment conducted in parentheses.
[b]Number of dead false positive in parentheses (see Figure S5).
Bia, Bialaphos; Hyg, hygromycin; ND, not determined; SD, standard deviation; UT-C, untransformed control (wild-type).

Southern blot hybridization. An autoradiogram of *Hin*dIII restriction enzyme-digested genomic DNA from hygromycin-tolerant and PCR-positive independent transformed lines after hybridization with the [α-^{32}P]-dCTP-labelled hptII gene fragment revealed that out of 11 randomly selected lines tested nine showed integration of single copy T-DNA (Figure 5d).

Segregation analyses showed a clear monogenic 3:1 ratio of resistant: susceptible of T2 progeny plants derived from self-fertilized single copy T-DNA integrated T1 plants (Table S2). We observed a significant level of expression of inherited reporter gene in the selected T2 lines using qRT-PCR (Figure 5d). A comparison of expression patterns among these transgenic lines revealed that reporter genes driven by the Ubi promoter had high levels of expression as compared with the CaMV35S promoter (Figure 5e). A flow chart for timeline of *S. viridis* spike-dip transformation procedure is given in Figure S6.

DISCUSSION

In the present study we standardized and optimized a rapid spike-dip method that facilitates high-throughput transformation of *S. viridis*, an emerging monocot model. While optimizing Arabidopsis floral-dip transformation, Clough and Bent (1998) suggested three critical requirements for successful transformation: (i) composition of infiltration medium; (ii) appropriate floral developmental stage and (iii) surfactant and/or vacuum to aid infiltration. In this study, we tested SvI, SvSD, MS, sucrose (5%) and MS + sucrose to identify a suitable medium composition for successful transformation. Using *A. tumefaciens* strain AGL1 carrying 35S::GUS reporter gene construct (Figure S1a) in a nutrient-rich SvSD medium, we found two- to four-fold higher transformation rates as compared with other media tested. A similar complex medium was used for the transient expression of Arabidopsis leaves by agroinfiltration (Lee and Yang, 2006). Keeping the major nutrient components of the induction medium reported by Lee and Yang (2006), we modified the sugar constituents to glucose (36 g L^{-1}) and sucrose (68.5 g L^{-1}) in our SvSD medium. This combination of glucose and sucrose was also found to be effective for high transformation of rice in tissue culture transformation methods (Mohanty *et al.*, 1999; Saha *et al.*, 2006b). In addition, Clough and Bent (1998) and Bent (2006) reported that 5% sucrose was suitable for successful transformation of Arabidopsis using floral dip. Although previously even though a SvI medium was found to be effective for the transient transformation of leaf cells of *S. viridis* (Brutnell *et al.*, 2010), we found that this medium was not appropriate for either transient or stable transformation of *S. viridis* using this method. We observed that both the simple SvI and MS media had no effect on transformation rates (Figure 1a). In addition, we did not see significant difference in transformation rates when we used 5% sucrose and MS + sucrose as an inoculation medium, which suggested that pH adjustments may not be a critical factor for successful transient or stable transformations. Supporting this notion, Clough and Bent (1998) also demonstrated that pH adjustments were unnecessary for successful transformation of Arabidopsis using floral dip.

Developmental stage of the flowers was shown to be crucial for successful floral transformations (Clough and Bent, 1998; Desfeux *et al.*, 2000; Trieu *et al.* 2000). We conducted an assessment of *S. viridis* accession A10.1 foxtail spikes developmental stage and categorized into seven stages (S1 to S7) of floral development, from early pre-anthesis to late post-anthesis spikes (Figure S2). It has been shown previously that high rates of transformation in Arabidopsis occurred when the plants had a maximum number of unopened floral bud clusters, and that the dipping of flowers later than 4 days before anthesis did not produce transformants (Clough and Bent, 1998). Here, we found that reproducible high-frequency transformation is

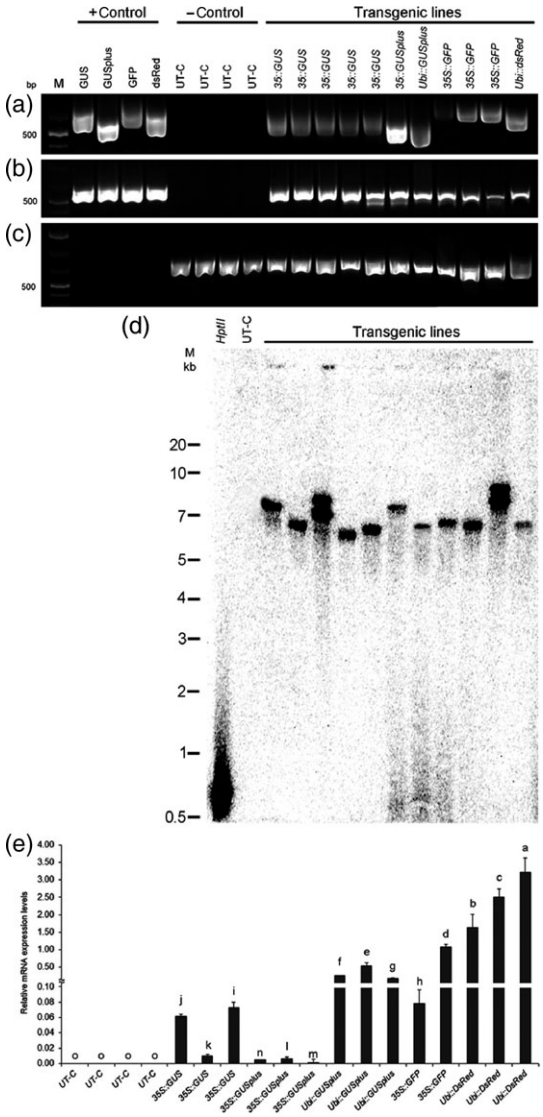

Figure 5. Molecular analyses of stable transformants from T1 and T2 generations.

(a–c) PCR analysis of putative T1 transformants for the presence of (a) reporter genes (GUS, GUSplus, GFP and DsRed), (b) selectable marker (HptII) gene, and (c) an endogenous TUB gene.

(d) Southern blot analysis of *Hin*dIII restriction enzyme-digested genomic DNA isolated from leaves of 11 independent T1 transgenic lines.

(e) Expression patterns of reporter genes (GUS, GUSplus, GFP and DsRed) in selected T2 lines as determined by qRT-PCR. M, Thermo Scientific O'Gene Ruler 1 kb Plus DNA ladder. Plasmid DNA of 35S::GUS, 35S::GUSplus, Ubi::GUSplus, 35S::GFP and Ubi::DsRed were used as positive (+) control while untransformed control (UT-C) plant DNA was taken as negative (−) control to amplify with each reporter gene-specific primer for PCR. Restriction enzyme digested genomic DNAs from T1 transgenic lines and UT-C (−control) including PCR purified HptII gene (+ control) were separated in a 1% agarose gel, and hybridized with a [α-^{32}P]-dCTP-labelled 578-bp HptII gene probe. Position of molecular weight markers are indicated on the left side. qRT-PCR results are presented as mean relative expression ($2^{-\Delta\Delta CT}$) with SE from three progeny plants of same T1 parent after normalization using an ACT endogenous reference gene. UT-C plant RNA was used as the negative (−) control to amplify with each reporter gene-specific primers for qRT-PCR. $^{a-o}$Different letters on the bars indicate significant differences at the $P \leq 0.05$ level as tested by the Tukey–Kramer HSD test. See Table S1 for primer sequences and product size.

achieved when the spikes were approximately 5.5 cm long over 6–8 days before anthesis at S3 (Figures 1 and S2). These results corroborate those found in wheat, in which 6–7 cm long spikes, which did not emerge from the sheath before 4–7 days of anthesis, were ideal for transformation (Zale *et al.*, 2009). Although a significant high transient GUS expression was seen at the early S2 stage, low stable transformants were obtained. In general, we noticed a gradual decrease in transformation rates with the progression of spike development towards anthesis (S4 to S6) (Figures 1c and S2). Moreover, a high histochemical GUS staining was seen at the bottom of each floret where the ovule is located (Figure 1b), indicating that the ovule is the probable target tissue amenable to T-DNA transfer upon *Agrobacterium* infection. In agreement with this notion, Bechtold and Pelletier (1998), Desfeux *et al.* (2000) and Ye *et al.* (1999) showed that the Arabidopsis ovule is the likely target for T-DNA transfer. To address the question of whether multiple *Agrobacterium* inoculations over the course of spike development might result in increased numbers of transformants, we performed three consecutive dipping of the same spikes on every alternate day. Multiple *Agrobacterium* inoculations caused bleaching of spikes with no seed setting as most plant tissues died.

Successful monocot transformation depends on the ability of the *Agrobacterium* cells to attach to the plant target cells (Shrawat and Lorz, 2006). We reasoned that increasing the infection time would allow a better access of *Agrobacterium* to the target tissues and result in higher transformation rates. Here, we determined that 20 min infection was the best time for an efficient spike-dip transformation. Furthermore, surfactants are known to function either as enhancers of cuticle penetration by making the plant cuticular membrane more susceptible to *Agrobacterium*, or by acting as co-solvents, thereby enhancing the movement of *Agrobacterium* into the plant cells (Madhou *et al.*, 2006). The supplementation of surfactants to improve the efficiency of the *Agrobacterium*-mediated genetic transformation of diverse plant tissues was apparent from previous studies (Clough and Bent, 1998; Cheng *et al.*, 2004; Zhang *et al.*, 2006; Kim *et al.*, 2009), although high surfactant levels in the infection medium could cause the necrosis of plant tissues (Clough and Bent, 1998). We determined that the rate of transformation did not change significantly when Tween 20 and Triton X-100 were used at concentrations between 0.01 and 1.0%. Conversely, Silwet L-77 at 0.025% produced about a four-fold higher stable transformants when spikes were inoculated with *Agrobacterium* stain AGL1 containing the 35S::GUS reporter gene (Figure 2b). Silwet L-77 is a preferred surfactant because of its low phytotoxicity, reduces surface tension compared with most surfactants and it improves penetration of bacteria into relatively inaccessible plant tissues (Whalen *et al.*, 1991). However, we observed that inoculation medium

containing 1.0% Silwet L-77 was deleterious because it caused necrosis of plant tissues.

The different *Agrobacterium* strains used in this study differed in their virulence, with strains EHA105 and AGL1 showing a superior transformation rate. Both EHA105 and AGL1 contain succinamopine type Ti-plasmid with C58 origin (Hamilton and Fall, 1971) and were shown previously to be suitable for monocot transformation (Hiei and Komari, 2008; Chen *et al.*, 2010).

Further to validate our transformation protocol and to examine the appropriate promoter to conduct future transformation in *S. viridis*, we conducted several transformations to overexpress GUSplus, GFP and DsRed driven by either CaMV35S or maize Ubi promoters. Chen *et al.* (2010) demonstrated that the GUSplus reporter gene was useful for high-throughput transient gene expression in switchgrass, while fluorescent (GFP and DsRed) reporter genes were known to be suitable for *Agrobacterium* transformations (Sheen *et al.*, 1995; Jach *et al.*, 2001). We monitored significantly higher expression of reporter genes in transient expression (Figure 3c,e) as well as subsequent T1 (Figure 4) and T2 (Figure 5e) generations when driven by the Ubi promoter. The maize Ubi promoter has been extensively employed for enhanced constitutive expression of target gene in monocot cereals (Toki *et al.*, 1992; Hiei *et al.*, 2014). Although the choice and the selection agent concentration are also important considerations when using this protocol, the concentration of selection agent should not be as high as to be lethal to low copy number T-DNA transformants (Wilmink and Dons, 1993). We chose HptII because it is a suitable selection marker with relatively low escapes from hygromycin antibiotic selection. The development of false positives in our study could be attributed to the stress induced by dipping, as similar results were reported in wheat by Zale *et al.* (2009), and demanded the use of a secondary screen. Thus the selection with a combination of hygromycin and bialaphos allowed us to identify putative transformants (Figures 5, S4 and S5). We further demonstrated the stable integration of the T-DNA cassette into the nuclear genome by PCR (Figure 5a,b) of genomic DNA isolated from hygromycin-resistant and GUS positive T1 lines (Figure 4 and Table 1) with respective primers specific for each reporter and selectable marker genes (Table S1). We confirmed the stable integration and determined the copy number of T-DNA by Southern blot analysis (Figure 5d). We also demonstrated a monogenic 3:1 Mendelian pattern of transgene inheritance (Table S2) and the expression of inherited transgenes in the subsequent T2 generation (Figure 5e).

In conclusion, we developed an alternative method for the *Agrobacterium*-mediated transformation of *S. viridis* using a spike dip that avoids the traditional *in vitro* culture steps. Several genotypes of *S. viridis* were transformed following the optimized conditions for transient gene expression assay as well as stable transformation. Fertile transgenic T1 plants can be obtained within 8–10 weeks and the expression and inheritance of transgenes can be monitored over generations. *In vitro* culture, plant tissue culture media, growth regulators and vacuum infiltration were not essential to obtain stable transformants at a rate of $0.8 \pm 0.1\%$ (Table 1), a transformation efficiency rate that is comparable to the reported transformation efficiency by floral dip in Arabidopsis (Clough and Bent, 1998). The application of the method reported here will facilitate the high-throughput transformation of *S. viridis*.

EXPERIMENTAL PROCEDURE

Plant materials and growth conditions

Three *Setaria viridis* (L.) Thell. genotypes, A10.1 (PI 669942/Ames 31045), 132 (PI Ames 28193) and 98HT-80 (PI 649320), were used in this study (Table 1). Seeds were obtained from the Germplasm Resources Information Network (GRIN, http://www.ars-grin.gov/), United States Department of Agriculture (USDA). Seeds from each genotype were sowed in germination trays (27.9×54.3 cm) (McConkey, Sumner, WA, USA) containing moist agronomy mix (equal parts of redwood compost, sand and peat moss) and vernalized at 4°C for 2 days in a Isotemp incubator (Fisher Scientific, Pittsburgh, PA, USA). Trays were kept in greenhouse at 28 ± 2°C with 50% relative humidity for a 16 h day/8 h night photoperiod for seed germination. After 7 days, seedlings were transferred to pots (10.2×8.2 cm) (McConkey) containing moist agronomy mix and allowed to grown under greenhouse conditions until flowering. Plants were watered every alternate day with deionized water and fertilized using a solution of 50% N:phosphorus:potassium (20:10:20) and 50% ammonium sulfate (total of 0.5 g of N) once in a week until spike initiation.

Transformation vectors and *Agrobacterium* strains

Five binary vectors, pCAMBIA1201, pH7m24GWp35S-GUSplus, pH7m24GW pUbi-GUSplus, pH7m24GW35Sp-GFP and pGWB17-UbiDsRED-UbiBAR, containing GUS (β-glucuronidase), GUSplus (β-glucuronidase plus), GFP and DsRed (*Discosoma* sp. red fluorescent protein) reporter genes were used in this study. The schematic representation of 35S::GUS, 35S::GUSplus, Ubi::GUSplus, 35S::GFP and Ubi::DsRed transformation vectors are shown in Figure S1. The reporter gene constructs were driven by either the cauliflower mosaic virus 35S (CaMV35S) or an intron-interrupted maize ubiquitin (Ubi) promoters, and CaMV35S or nopaline synthase (NOS) terminators within the right (RB) and left (LB) borders of the T-DNA cassette region. These binary vectors contain HptII (hygromycin phosphotransferase II) and Bar (bialaphos) genes as plant selection markers. Four *Agrobacterium tumefaciens* strains used were AGL1(Lazo *et al.*, 1991), EHA105 (Hood *et al.*, 1993), GV3101 (Koncz and Schell, 1986) and LBA4404 (Hoekema *et al.*, 1983). The plasmids for each set of experiments were electrotransformed into the respective *A. tumefaciens* strains using a Gene Pulser Xcell™ Electroporation Systems (Bio-Rad, Hercules, CA, USA) following the method described before (Lin, 1995).

Bacterial culture and dipping medium

A single colony of each *A. tumefaciens* strain harboring one of the five binary vectors was cultured overnight in 5 ml liquid YEB medium (Vervliet *et al.*, 1975), containing appropriate antibiotics, at

28°C with an agitation of 250 rpm. The following day, 500 μl of this starter culture was added to 50 ml of culture medium containing 5 g L^{-1} tryptone, 2.5 g L^{-1} yeast extract, 5 g L^{-1} NaCl, 5 g L^{-1} mannitol, 100 mg L^{-1}, $MgSO_4$, 250 mg L^{-1} K_2HPO_4, 1.2 g L^{-1} glutamic acid, 15 g L^{-1} sucrose, pH 7.2 and antibiotics, and the bacteria was grown for 16 h at the same conditions as mentioned above. The next day, after adjusting the OD_{600} of the culture to 1.0, the culture was collected by centrifugation at 2400 ***g*** for 5 min and re-suspended in dipping medium.

The five dipping media tested were as follows: (i) *Setaria viridis* Infiltration (SvI) medium consisting of 50 mM (*N*-morpholino) methanesulfonic acid (MES), 2 mM Na_3PO_4, $12H_2O$, 0.025% (w/v) glucose; pH 5.8 (Brutnell *et al.*, 2010); (ii) *Setaria viridis* spike-dip (SvSD) medium composed of 10.5 g L^{-1} K_2HPO_4, 4.5 g L^{-1} KH_2PO_4, 1 g L^{-1} $(NH4)_2SO_4$, 0.5 g L^{-1} Na citrate, 4 g L^{-1} glycerol, 1 mM $MgSO_4$, 15 g L^{-1} ascorbic acid, 10 mM MES; pH to 5.8 modified from Lee and Yang (2006) and supplemented with 68.5 g L^{-1} sucrose, 36 g L^{-1} glucose (Mohanty *et al.*, 1999); (iii) MS full strength basal medium; pH 5.8 (Murashige and Skoog, 1962); (iv) sucrose (5% w/v) (Bent, 2006); (v) MS full strength basal medium fortified with 5% (w/v) sucrose; pH 5.8 (Clough and Bent, 1998).

Agrobacterium-mediated transformation and selection of putative transformants

Prior to spike dip, the bacteria harboring the reporter gene construct were re-suspended in 40 ml dipping medium supplemented with 200 μM acetosyringone (PhytoTechnology Laboratories, Overland Park, KS, USA) in a 50 ml Falcon tube and pre-induced by shaking at 180 rpm for 1 h. Plants bearing spikes at suitable developmental stage (Figure S2) were labelled (Figure S3a) and 15 min before to transformation all spikes were made wet by applying 1 ml of infiltration solution (10 mM $MgSO_4$, 10 mM MES; pH to 5.8) (Lee and Yang, 2006) containing Silwet-L77 (Lehle Seeds, Round Rock, TX, USA) in the range of 0.01–0.10% (v/v). Inflorescences were then immersed into the pre-induced *Agrobacterium* culture for 10–40 min with an occasional gentle agitation at 5-min intervals (Figure S3b). After dipping, plants were kept under the dome at low light intensity for overnight at 22°C. Next day plants were returned at 25°C for 16 h day/8 h night photoperiod with 50% relative humidity in the growth chamber or in the growth room where they grew for 5–7 days, and during this time transient transformation assay was performed on three or five DPD spikes. Finally, the plants were put inside the arecones (arabase and aratubes, Lehle Seeds) and grown under greenhouse conditions mentioned above for 2–4 weeks until maturity, when spikes turned brown and dry, and set seeds (Figure S3c). Seeds were harvested by gently removing the arecones and collected in microfuge tubes and stored at 4°C under desiccation.

Seeds obtained from T1 plants and subsequent generations were dehusked, and surface sterilized using 10% commercial bleach with 0.1% Tween 20 for 5 min followed by three to five washes in sterile deionized water. Seeds were blotted dry on sterile filter papers for 5 min and 20–25 seeds were transferred to plates containing half strength MS medium fortified with either 30 mg L^{-1} hygromycin (PhytoTechnology Laboratories, Shawnee Mission, KS, USA) or 3 mg L^{-1} bialaphos (PhytoTechnology Laboratories*)* as described before (Saha *et al.*, 2006b). Petri plates and lids were sealed with parafilm tape and kept at 4°C for 2 days for seed vernalization. Plates were then kept horizontally and incubated in the growth chamber (Percival Scientific, Perry, IA, USA) at 25°C with 16 h day/8 h night photoperiod and 50% relative humidity. Putative transformants were identified as hygromycin-resistant seedlings with well-developed green leaves showing profuse rooting after 10 DPG for selection on hygromycin (Figures S4 and 5). Selected putative transformants were transferred to soil in pots and grown under greenhouse conditions until maturity. The percentage (%) of transformation efficiencies were calculated as (number of hygromycin-resistant seedlings)/(total number seeds tested) × 100.

β-Galactosidase activity (GUS) assay and fluorescence microscopy

Histochemical staining and fluorometric measurements of the β-galactosidase (GUS) activity were performed according to the method described by Jefferson *et al.* (1987). The transformed tissues were soaked in the GUS staining buffer (100 mM sodium phosphate, 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 10 mM Na_2EDTA; pH 7.0) and incubated for 16 h at 37°C. Next day, the stained tissues were washed by repeated rinsing with 3:1 (v/v) absolute alcohol: acetic acid until completely cleared the chlorophylls and photographed.

For fluorometric measurements of the GUS activity, transformed spikes after dipping were homogenized in the extraction buffer (50 mM sodium phosphate, 10 mM Na_2EDTA, 0.1% Triton X-100, 0.1% sarcosyl, 10 mM β-mercaptoethanol; pH 7.0) and centrifuged for 15 min at 16 100 ***g*** to collect the supernatants. The protein concentrations were determined following the protocol described before Bradford (1976) using bovine serum albumin (BSA) as standard. The GUS activity was carried out using 10 μg of total protein after incubation with the substrate 4-methylumbelliferyl-β-D-glucuronide (4-MUG; Sigma, St. Louis, MO, USA) for 1 h at 37°C. The reaction was terminated by adding 0.2 M Na_2CO_3, and the fluorescence was measured at 365 nm excitation and 455 nm emission using a 96-well BioTek SynergyMx microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Specific GUS enzyme activity was determined based on the standard curve of 4-methylumbelliferone (4MU; Sigma) standards from the same microtiter plate. Data were recorded using Gen5 (version 1.11.5) software (BioTek) and relative GUS activity in each sample was calculated after normalization of the fluorescence signal value by subtracting of the background fluorescence signal detected from equal amount of proteins in the mock control.

Expression of GFP and red fluorescent protein (DsRed) was observed using a Leica MZFLIII fluorescence stereomicroscope (Leica Microsystems Inc., Buffalo Grove, IL, USA) equipped with filters for GFP (excitation 395 nm and emission 509 nm) and DsRed (excitation 554 nm and emission 586 nm) respectively, and a SPOT Insight charge coupled device (CCD) camera. Photographs were captured using SPOT Advanced (version 4.1) software (Diagnostic Instruments, Inc., Sterling Heights, MI, USA).

Nucleic acids analysis

Genomic DNA was isolated from transformed and untransformed control tissue followed by the cetyltrimethylammonium bromide (CTAB) extraction method (Doyle and Doyle, 1987). PCR analysis for detection of the transgenes (GUS, GUSplus, GFP, DsRed, HptII and Bar) was carried out as described before (Saha *et al.*, 2006a) using pair of primer specific for each gene (Table S1). The five binary vector plasmids were used as a positive control for PCR. The amplicons were separated by electrophoresis on 1% (w/v) agarose gel.

Southern blot analysis was carried out according to Sambrook *et al.* (1989). Genomic DNA (10 μg) from transformed and

untransformed control plants were digested with *Hin*dIII (New England BioLabs, Inc., Ipswich, MA, USA) restriction enzyme, fractionated on a 1% (w/v) agarose gel, denatured and transferred onto a N + -nylon membrane (Amersham Biosciences, GE Healthcare Life Sciences, Pittsburgh, PA, USA). Hybridization of membrane with the [α-^{32}P] dCTP (Molecular Probe, Life Technologies) labelled PCR amplified HptII gene probe, followed by washings under stringent conditions, was carried out according to procedures described earlier (Saha *et al.*, 2007). Blot was exposed to phosphor imaging screen (Molecular Dynamics, GE Healthcare Life Sciences, Pittsburgh, PA, USA). Phosphor images were scanned on a Typhoon 8600 Variable Mode Imager (Molecular Dynamics, GE Healthcare Life Sciences, Pittsburgh, PA, USA) and the scans were analyzed using Typhoon Scanner Control (version 1.0) software (GE Healthcare Life Sciences). Image QuantTL Array (version 8.1) software (GE Healthcare Life Sciences) and Adobe Photoshop CS5 (version 12.0) software were used to adjust the digital image.

For quantitative real-time PCR (qRT-PCR), gene-specific primer pair was designed using the Primer Express (version 3.0) software (Applied Biosystems, Foster City, CA) following the default parameters according to the manufacturer's recommendation (Table S1). Total RNA was isolated from 100 mg of frozen ground tissue using the RNeasy plant mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's procedure. The RNA sample was assessed by NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) and the first strand cDNA synthesis was carried out according to the protocol described earlier (Saha and Blumwald, 2014) using the QuantiTect reverse transcription kit (Qiagen). The qRT-PCR reactions were performed in a reaction volume of 5 μl containing 1 μl of diluted cDNA, 200 nM of each gene-specific primer and 2.5 μl of 2× Fast SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in an optical 96 well plate (Applied Biosystems) using a StepOnePlus™ Real-Time PCR System (Applied Biosystems) equipment as previously described (Saha *et al.*, 2013). An ACT (actin) gene was used as an endogenous reference gene (Table S1) to normalize gene expression and the fold change of transgenes expression were calculated by the $2^{-\Delta\Delta CT}$ equation as described before (Saha *et al.*, 2013).

Segregation analysis

Segregation analysis of T2 seeds was carried out according to Dutta *et al.* (2005) on hygromycin (30 mg L^{-1}) amended half-strength MS medium and 10 DPG seedling were scored as hygromycin resistance (Hyg^R) or hygromycin sensitive (Hyg^S).

Statistical analysis

All experiments were repeated three times. Mean with SD for each experiment was calculated using Microsoft Excel (version 2010) software. Tukey–Kramer honest significant difference (HSD) test was performed for multiple comparisons to evaluate significant differences at the $P \leq 0.05$ level within experiments using JMP (version 7.0.2) software (SAS Institute Inc. Cary, NC, USA). Segregation patterns of the T2 progeny plants were validated using the chi-squared test.

ACKNOWLEDGEMENTS

Authors are thankful to Dr Ellen Tumimbang, Dr Hiromi Tajima, Elham Abed, Yrian Hong and Hye Won Kim for technical support. This work is funded by the United States Agency for International Development (USAID) to support the Feed the Future Innovation Laboratory for Climate-Resilient Millet under Grant No. APS M/OAA/GRO/EGAS-11–002011.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Schematic representation of a linearized map of plant transformation vectors.

Figure S2. Developmental stages of *S. viridis* accession A10.1 foxtail spikes.

Figure S3. Steps of spike dip transformation.

Figure S4. Selection of putative transformants at the T1 plant generation.

Figure S5. Representation of stable transformants and hygromycin susceptible false positive seedlings after 10 DPG growing on half MS + hygromycin (30 mg L^{-1}) plates and hygromycin-resistant putative transformed seedlings with well-developed green leaves showing profuse rooting in the presence of hygromycin.

Figure S6. Flow chart for timeline of *S. viridis* spike dip transformation procedure.

Table S1 Details of PCR and qRT-PCR primer sequences used for *S. viridis* spike dip method.

Table S2 Segregation analyses of T2 progeny plants derived from self-fertilized T1 plants.

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