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Letter to the Editor

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Engineering Non-transgenic Gynoecious Cucumber Using an Improved Transformation Protocol and Optimized CRISPR/Cas9 System

Dear Editor,

Gynoecism has been extensively exploited in cucumber breeding. The utilization of a gynoecious line permits earlier production of hybrids, higher yield, and more concentrated fruit set. In addition, the utilization of a gynoecious line eliminates the need for hand emasculation and reduces the labor cost of crossing (Robinson, 2000). Therefore, the development of gynoecious inbred lines is instrumental for cucumber breeding. Gynoecious inbreds can be produced by selection from crosses of monoecious inbreds, or can arise spontaneously from natural variation. However, both methods have disadvantages. For instance, the time-consuming and laborious process of crossing can also lead to the introduction of undesirable traits, and spontaneous evolution of gynoecious varieties may not occur in lines of interest for breeders. *CmWIP1* acts as an inhibitor of carpel development, and mutation of *CmWIP1* confers a gynoecious phenotype in melon (Martin et al., 2009). Modification of the *CmWIP1* ortholog in cucumber, *CsWIP1*, may accelerate the development of gynoecious inbred lines. However, cucumber is intractable to transformation. The low efficiency of transformation in cucumber makes it a daunting task to apply gene editing tools such as CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated system 9). To date, CRISPR/Cas9 gene editing has been reported only once in cucumber (Chandrasekaran et al., 2016). In that case, disruption of *eIF4E* (*eukaryotic translation initiation factor 4E*) led to broad viral resistance; however, mutation in *eIF4E* was detected in only one of five T0 plants, indicating low efficiency of gene editing. In this study, we aimed to establish an improved transformation protocol for cucumber and to generate a gynoecious cucumber line through CRISPR/Cas9-mediated mutagenesis of *CsWIP1*.

To improve the genetic transformation efficiency of cucumber, green fluorescent protein (GFP) was used as a reporter during *Agrobacterium*-mediated infection and plant regeneration. Cotyledonary nodes were used as explants, which were immersed in *Agrobacterium* solution and subsequently co-cultivated for 3 days. Only weak GFP fluorescence was observed in infected explants, indicating insufficient *Agrobacterium* infection (Figure 1A). Regeneration of infected explants yielded GFP-negative shoots and brightly fluorescing calli that were unable to form adventitious buds (Supplemental Figure 1A). This observation suggested that *Agrobacterium* infection may fail to extend to cells from which adventitious buds originate. A cell population that expresses the meristem marker gene *SHOOT MERISTEMLESS* (*STM*) is responsible for axillary meristem initiation in leaf axils (Shi et al., 2016). *In situ* hybridization assays showed that *CsSTM* was strongly expressed in shoots that regenerated from cells in the deep layers of the U-shaped cut end that is produced when generating explants (Figure 1B; Supplemental Figure 1B).

We speculated that if cells of the deeper layers were infected, transgenic shoots could be obtained. A physical method using vacuum infiltration was previously developed to enhance *Agrobacterium* infection and successful transformation has been achieved by using an F1 hybrid (Nanasato et al., 2013). Nevertheless, transformation has not been widely adopted in cucumber research, probably because vacuum pumps can be cumbersome to use, and many lines of interest are inbred lines. In this study, the vacuum pump was replaced with a simple syringe for vacuum infiltration (Supplemental Figure 2A). Examination of GFP fluorescence after co-cultivation showed that the region and intensity of the fluorescent signal was different between vacuum infiltration and immersion. Under vacuum infiltration, the GFP signal was both stronger and found in the deeper cell layers of explants (Figure 1A). The frequency of fluorescent explants was also increased by vacuum infiltration (Supplemental Figure 2B). After cultivation for 2 weeks in regeneration medium, GFP-positive buds emerged (Figure 1C and Supplemental Figure 3A and 3B). Fluorescent shoots were separated from the explants and elongated (Figure 1C).

To promote rooting, low concentrations of auxin were generally used. However, the addition of auxin appeared to promote chlorosis of the regenerated shoots, which may be caused by auxin-stimulated ethylene production (Supplemental Figure 3C). It was previously reported that CO signaling acts downstream of auxin in the adventitious rooting process in cucumber (Xuan et al., 2008). Hemin, a heme-oxygenase activator/CO donor that can increase the CO concentration, was used for rooting. As shown in Supplemental Figure 3C, supplementing the medium with hemin induced rooting, and shoots also appeared healthier.

The resultant T0 transgenic plants were maintained in a climate-controlled chamber (Supplemental Figure 4A). As expected, GFP fluorescence was detected in tendrils, male flowers, and ovaries of T0 plants (Figure 1D and Supplemental Figure 4B and 4C). In total, three independent T0 transgenic lines were generated from 1132 seeds. The transformation efficiency was 1.32‰. The same method was applied to another Cucurbitaceae species, melon, except that the concentration of 6-benzylaminopurine was reduced to 0.5 mg/l. Three GFP-positive transgenic melon plants were obtained from 1400 seeds; fluorescent ovaries are shown in Supplemental Figure 4D. These results demonstrate

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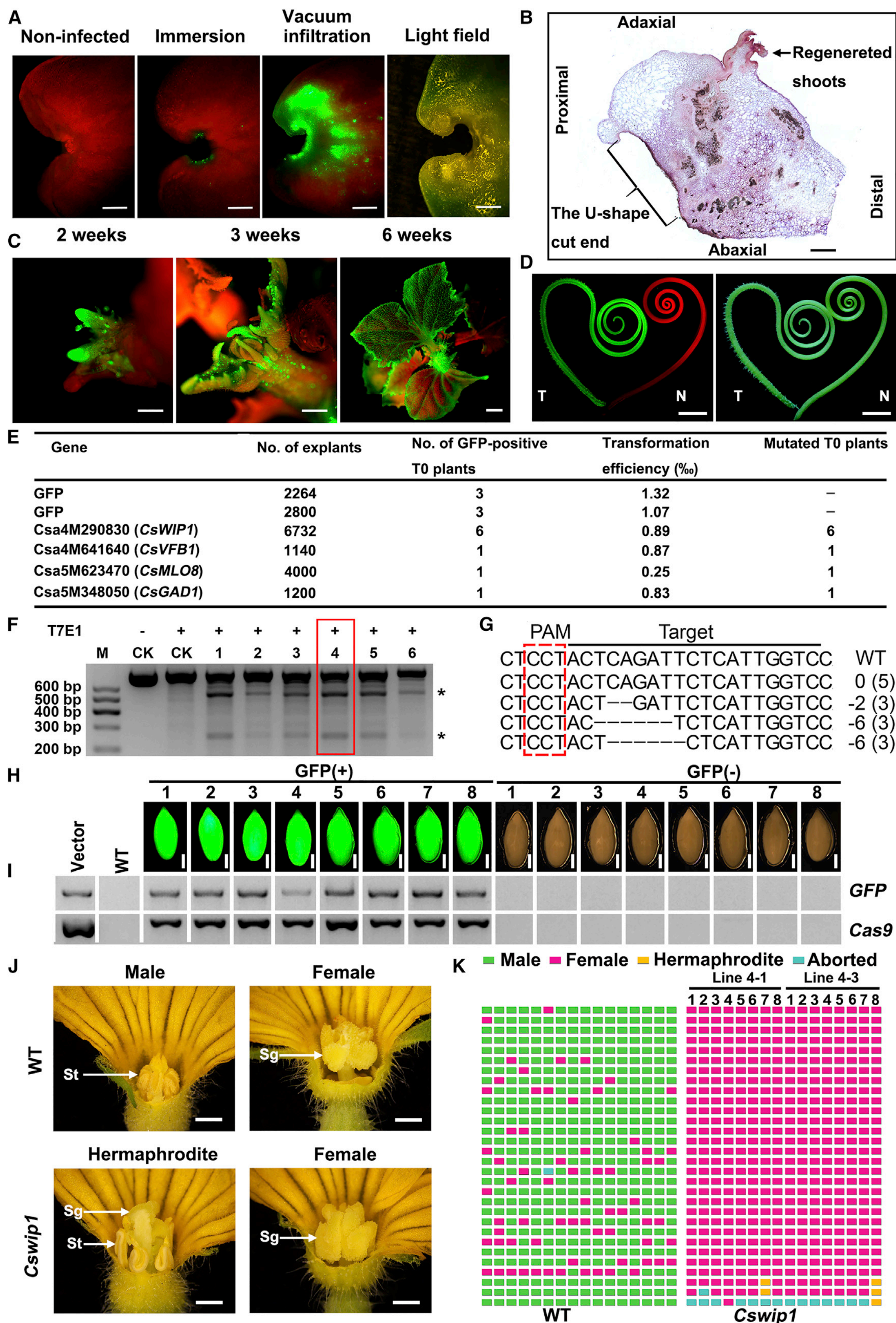

Gene	No. of explants	No. of GFP-positive T0 plants	Transformation efficiency (‰)	Mutated T0 plants
GFP	2264	3	1.32	–
GFP	2800	3	1.07	–
Csa4M290830 (*CsWIP1*)	6732	6	0.89	6
Csa4M641640 (*CsVFB1*)	1140	1	0.87	1
Csa5M623470 (*CsMLO8*)	4000	1	0.25	1
Csa5M348050 (*CsGAD1*)	1200	1	0.83	1

(legend on next page)

that the protocol established in this study can be widely used in Cucurbitaceae species with a transformation efficiency approaching 1‰.

The efficiency of the CRISPR/Cas9 system is largely dependent on the level of sgRNA expression (Ma et al., 2016). Although the *Arabidopsis thaliana U6* (*AtU6*) promoter is sufficient to generate high sgRNA levels in most cases, an endogenous U6 promoter may drive even higher levels, with a subsequent positive influence on mutation frequency. To drive high-level expression of sgRNA, the pHSE401 and pKSE401 constructs were modified to include the endogenous *U6* promoter (Xing et al., 2014) (Supplemental Figure 5A). Four *CsU6* promoters were compared for targeted mutagenesis in cucumber callus using a T7 Endonuclease I (T7EI) assay and Sanger sequencing (Supplemental Figure 5B and 5C). The four *CsU6* promoters induced mutations at different rates: 65.2% for *CsU6-1*, 57.8% for *CsU6-2*, 24.1% for *CsU6-3*, and 61.1% for *CsU6-4*. Given the high mutation efficiency achieved with *CsU6-1*, it was selected for further experiments (Supplemental Figure 5A and 5C). In addition, to facilitate the selection of positive transformants and their subsequent transgene-free mutant progeny, a GFP cassette was cloned into the CRISPR/Cas9 vectors pHCG401 and pKCG401, which contain the *CsU6-1* promoter, enabling constitutive GFP expression in transgenic plants.

To assess our protocol for transformation and genome editing, we targeted *CsWIP1* (*Csa4M290830*), *CsVFB1* (*Csa4M641640*), *CsMLO8* (*Csa5M623470*), and *CsGAD1* (*Csa5M348050*) for mutation. The transformation efficiency approached around 1‰ (Figure 1E). Editing at the desired sites was detected in all T0 plants by the T7E1 assay and Sanger sequencing (Figure 1F and Supplemental Figure 6A–6F), indicating a higher mutation efficiency than previously reported (Chandrasekaran et al., 2016). *Csvfb1* T0 mutants formed smaller leaves with smooth leaf margins in contrast to wild-type plants (non-transgenic CU2), which had larger leaves with serrations at the margin (Supplemental Figure 6G). *Cswip1* T0 mutants displayed a gynoecious phenotype, with the upper nodes bearing only female flowers (Supplemental Figure 6H), indicated that *CsWIP1* acts as an inhibitor of carpel development in cucumber, as *CmWIP1* does in melon.

To generate transgene-free gynoecious cucumber, the *Cswip1* T0 plant line 4, which displayed a high mutation rate, was chosen for mutation analysis and crossing. Three types of deletions were introduced in *CsWIP1*, and the mutation rate was 64.3% (Figure 1G). Sequencing PCR products of potential off-target sites detected no mutations (Supplemental Figure 7). T1 seeds were obtained by cross-pollinating with wild-type. Among 214 T1 seeds, 98 were GFP positive and 116 were GFP negative (Figure 1H). PCR analysis demonstrated that *Cas9* and *GFP* co-segregated (Figure 1I). The segregation of transgenic and non-transgenic plants in the T1 population approached 1:1, indicative of a single copy insertion. We used the co-segregation of *GFP* and *Cas9* to facilitate screening of transgene-free mutants by selecting GFP-negative seeds. We sequenced 34 of the GFP-negative T1 plants, all of which were heterozygous mutants that each carried one of the mutations (Supplemental Figure 8), indicating that *CsWIP1* were bi-allelic disrupted in line 4 before fertilization. This high mutation frequency may be caused by the continuing activity of Cas9/sgRNA. The heterozygous T1 mutants were self-pollinated, and homozygous, transgene-free *Cswip1* T2 mutants were obtained through PCR genotyping and sequencing (Supplemental Figure 9). *Cswip1* mutants bore female and hermaphroditic flowers instead of male and female flowers in the wild-type (Figure 1J and Supplemental Figure 10A). Compared with monoecious wild-type plants, which bear four female flowers on average, the *Cswip1* mutant had seven times more female flowers than wild-type (Figure 1K and Supplemental Figure 10B). Fruit produced from the hermaphroditic flowers of *Cswip1* were short and round, whereas fruit from female flowers were indistinguishable from fruit produced by the wild-type (Supplemental Figure 10C).

In conclusion, we report a simplified and more effective transformation protocol for cucumber and melon. We found that vacuum infiltration with a syringe promotes *Agrobacterium* infection of the cells from which transgenic shoots regenerate. In addition, the use of a CO donor, hemin, reduces chlorosis and accelerates rooting of regenerated shoots. We successfully obtained transgenic

Figure 1. Engineering Non-transgenic Gynoecious Cucumber by Genome Editing.

(A) Enhanced *Agrobacterium* infection by vacuum infiltration. From left to right, the first three images were taken under the GFP channel of a fluorescence microscope. The fourth is a bright-field image of the U-shaped cut end of a cotyledon node explant. Scale bar, 1 mm.

(B) *In situ* hybridization with the CsSTM antisense probe. Scale bar, 0.5 mm.

(C) Regenerated shoots displaying GFP fluorescence emerged successively in regeneration medium. Scale bars: left and middle, 1 mm; right, 2 mm.

(D) GFP-fluorescent tendrils of the transgenic T1 plant. The left image was taken under the GFP channel. The right image was taken under the bright-field channel. T, transgenic; N, non-transgenic. Scale bar, 5 mm.

(E) Summary of transformation and gene editing efficiency in T0 transgenic plants.

(F) T7EI analysis of CsWIP1 target mutation in six T0 transgenic plants. PCR fragments of the wild-type were used as control (CK). The plus and minus signs indicate the presence or absence of the T7E1 enzyme. Line 4 is highlighted with a red rectangle. Stars represent the cleaved bands. M, DNA marker.

(G) Sanger sequencing analysis of mutant alleles in transgenic line 4. The PAM sequence is highlighted in the red rectangle and the target sequence is indicated by the black line. Nucleotide deletions are shown by dashes, and deletion sizes (nucleotides) are indicated on the right. Numbers in parentheses represent the number of colonies detected by sequencing.

(H) GFP fluorescence of the T1 progeny seeds. GFP(+) represent GFP-positive seeds and images were taken under the GFP channel; GFP(-) represent GFP-negative seeds and images were taken in the bright field. Scale bar = 2 mm.

(I) Amplification of GFP and Cas9 fragments in 16 T1 plants. The pKCG401 vector was used as a positive control and the WT genomic DNA was used as negative control.

(J) Flower types from a monoecious plant (WT) and Cswip1 transgene-free T2 mutants. Sg, stigma; St, stamen. Scale bar, 1 cm.

(K) Graph of flower phenotypes of WT and Cswip1 T2 mutants. Sixteen T2 individuals from the progeny of line 4-1 and line 4-3 represent the transgene-free Cswip1 mutants. Each column represents an individual plant, and each rectangle represents a node. The first 30 nodes of each plant are shown.

cucumber using an inbred line rather than an F1 hybrid as previously used (Nanasato et al., 2013). We further optimized the CRISPR/Cas9 system by using stronger *CsU6* promoter and a GFP tag to facilitate selection both the transformants and transgene-free mutants among the progeny. With these optimized procedures, we generated transgene-free gynoecious cucumber plants from a commercially valuable inbred line, which will be useful for heterosis breeding. Future efforts to further improve the transformation efficiency could focus on exploiting regeneration-promoting genes, such as *WUS* and *STM* (Lowe et al., 2016).

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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AUTHOR CONTRIBUTIONS

L.Y., B.H., D.L., J.S., and S.H. conceived and designed the experiments, B.H., L.Y., D.L., X.L., J.Q. and S.Z. performed the experiments, L.Y., B.H., D.L., D.G., and J.S. analyzed the data, and D.G and L.Y. wrote the manuscript.

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