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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that the protocol established in this study can be widely used in Cucurbitaceae species with a transformation efficiency approaching 10^5.

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), CsML08 (Csa5M624370), and CsGAD1 (Csa5M348050) for mutation. The transformation efficiency approached around 10^6 (Figure 1E). Editing at the desired sites was detected in all T0 plants by the T7EI assay and Sanger sequencing (Figure 1F and Supplemental Figure 6A–6F), indicating a higher mutation efficiency than previously reported (Chandrasekaran et al., 2016). Csfvb1 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...
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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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