Development of an Agrobacterium-Mediated Stable Transformation Method for the Sensitive Plant *Mimosa pudica*

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**Abstract**

The sensitive plant *Mimosa pudica* has long attracted the interest of researchers due to its spectacular leaf movements in response to touch or other external stimuli. Although various aspects of this seismonastic movement have been elucidated by histological, physiological, biochemical, and behavioral approaches, the lack of reverse genetic tools has hampered the investigation of molecular mechanisms involved in these processes. To overcome this obstacle, we developed an efficient genetic transformation method for *M. pudica* mediated by *Agrobacterium tumefaciens* (Agrobacterium). We found that the cotyledonal node explant is suitable for Agrobacterium-mediated transformation because of its high frequency of shoot formation, which was most efficiently induced on medium containing 0.5 μg/ml of a synthetic cytokinin, 6-benzylaminopurine (BAP). Transformation efficiency of cotyledonary node cells was improved from almost 0 to 30.8% positive signals arising from the intron-sGFP reporter gene by using Agrobacterium carrying a super-binary vector pSB1111 and stabilizing the pH of the co-cultivation medium with 2-(N-morpholino)ethanesulfonic acid (MES) buffer. Furthermore, treatment of the explants with the detergent Silwet L-77 prior to co-cultivation led to a two-fold increase in the number of transformed shoot buds. Rooting of the regenerated shoots was efficiently induced by cultivation on irrigated vermiculite. The entire procedure for generating transgenic plants achieved a transformation frequency of 18.8%, which is comparable to frequencies obtained for other recalcitrant legumes, such as soybean (*Glycine max*) and pea (*Pisum sativum*). The transgene was stably integrated into the host genome and was inherited across generations, without affecting the seismonastic or nyctinastic movements of the plants. This transformation method thus provides an effective genetic tool for studying genes involved in *M. pudica* movements.

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**Introduction**

Being fixed in the soil, rooted plants have evolved a variety of strategies to survive stressful environments. Despite lacking muscular and nervous systems, which play pivotal roles in animal motility, certain plant species have acquired the ability to undergo rapid leaf movements in response to external stimuli [1]. The compound leaves of the leguminous species *Mimosa pudica* exhibit seismonastic movement within seconds [2] of being touched or subjected to other types of stimulation [3]. This rapid movement has been suggested to reduce predation risks [4] by scaring away predators [1], decreasing the visibility of the leaves [1], or exposing protective thorns that are usually obscured behind the leaves [5]. The physiological mechanisms underlying seismonastic movement have been studied extensively since the 19th century [6]. This movement is caused by a loss of turgor pressure in one half of the pulvinus (extensor [7]), which is located at the base of each primary petiole, pinna, and pinnule (leaflet) [6]. Nuclear magnetic resonance (NMR) imaging demonstrated that water is translocated from the extensor half to the other half (flexor [7]) of the pulvinus during the movement [8]. At the cellular level, individual “motor cells” in the extensor half of the pulvinus shrink following outflow of intracellular water [9,10], which is accompanied by a large efflux of K⁺ and Cl⁻ ions [11,12,13,14]. These rapid movements of water and ions are difficult to explain by a simple diffusion model [12,15], suggesting that special mechanisms, such as solute-water co-transporters or contractile proteins, are involved in this process [15]. Pharmacological and cytological studies indicate that fragmentation of the actin cytoskeleton [16,17], depolymerization of its tyrosine residues [17,18], and changes in Ca²⁺ level [10,19] in pulvinar motor cells participate in the movement. The seismonastic reaction can be propagated over a distance by an electrical action potential [20], which is likely transmitted through the protoxylem [20,21] and the phloem [22]. Chemical substances also contribute to the long-range transmission of the movement [23] and several candidate substances were identified...
by chemical analysis and bioassays [24,25]. Mechanoreceptor cells in *M. pudica* have long been enigmatic; however, a recent study identified such cells on the tertiary pulvinus [26]. Despite many advances in our understanding of the physiology of seismosensitive movements in *M. pudica*, the genetic mechanisms underlying this phenomenon remain to be unraveled, due to the lack of reverse genetic tools for this species. Until now, there was no technique for introducing desired genes into this plant's genome. Agrobacterium-mediated genetic transformation is widely used to generate transgenic plants [27] and is a well-established technique in model legumes such as *Medicago truncatula* [28] and *Lupinus japonicus* [29]. However, transformation of other “recalcitrant” legumes, including *M. pudica*, remains challenging, because of the low frequency of shoot formation *in vitro* and the difficulty in transferring genes to cells that are capable of forming shoots [30,31,32].

In the present study, we developed an efficient Agrobacterium-mediated transformation method in *M. pudica*. To overcome the obstacles described above, we examined the shoot formation frequency of several types of explants and selected the cotyledonary node explant, which formed shoots at the highest frequency among the explants tested, as the starting material. We found that a super-binary vector, pSB111 [33], which exhibits improved transformation efficiencies due to the presence of additional virulence genes in the vector backbone [34], increases the number of transformed cells on the cotyledonal node. Furthermore, we demonstrated that controlling the pH during co-cultivation is required for efficient transformation. We thus established an effective transformation method for *M. pudica* that can be used to conduct reverse-genetic studies on the seismosensitive movements of this plant.

**Materials and Methods**

**Construction of T-DNA vectors and preparation of Agrobacterium cells**

A DNA fragment containing the coding sequence of synthetic green fluorescent protein (sGFP) [35] was PCR amplified from pUGW4 [36] using a pair of primers (5’-AAAGT CGACT CTTGA GCAAG GCGCA GGAG-3’ and 5’-TTGAG CTTGG ACTCTG TCCGT CCATG C-3’) and subcloned into the pCR-Blunt II-TOPO vector (Life Technologies, Carlsbad, USA). A DNA fragment containing the first intron of the castor bean beta-glucuronidase gene ([37] was amplified from pIG121-Hm [38] with primers (5’-CTAAG CTTCG CAAGA CCCTT CCTC-3’ and 5’-ATTTC ACGGG TTGGG GTTTC TACAG GACG T-3’) and inserted into the pCR-Blunt II-sGFP construct. Then a DNA fragment containing the intron-sGFP region was excised by digestion with XhoI and XbaI, and inserted into the SacI/XbaI site of the pCR-Blunt II-sGFP construct. Then a DNA fragment containing the intron-sGFP region was excised by digestion with SacI and XbaI, and inserted into the SacI/XbaI site of pIG121-Hm to produce the pIF121-Hm vector, in which the coding sequence of the beta-glucuronidase gene (uidA) [39] was replaced by that of sGFP. pIF121-Hm was then introduced into four different *Agrobacterium* strains (AGL1, GV2260, AGL1-G400, and GV3101) by electroporation and the pSB111-GFP vector was then produced by homologous recombination between pSB1 and pSB111-GFP in Agrobacterium. Agrobacterium cells harboring pIF121-Hm or pSB111-GFP were selected on LB medium containing 50 μg/ml hygromycin B (Life Technologies) and stored as glycerol stocks at −80°C.

**Preparation of cultivation media**

Germination medium (GM) consisted of half-strength basal MS salts (1/2 MS; Wako, Osaka, Japan) and 0.2% (w/v) gellan gum (Phytagel; Sigma-Aldrich, St. Louis, USA) at pH 5.8. Shoot induction medium (SIM) consisted of 1/2 MS, 2% (w/v) sucrose, 1× Gamborg’s vitamins (Sigma-Alrich), 0.5 μg/ml 6-benzylaminopurine (BAP; Sigma-Aldrich), and 0.3% gellan gum at pH 5.8. Selection medium (SEM) was prepared by supplementing SIM with 15 μg/ml hygromycin B (Sigma-Aldrich) and 150 μg/ml cefotaxime sodium salt (Sanofi K.K., Tokyo, Japan). Co-cultivation medium (COM) consisted of 1/2 MS, 2% sucrose, 1× Gamborg’s vitamins, 0.5 μg/ml BAP, and 0.1% (w/v) 2-(N-morpholino)ethanesulfonic acid (MES; Dojindo Laboratories, Kamimashiki-gun, Japan) at pH 6.1 or other values as indicated in the text. Each cultivation medium was prepared as follows: MS basal salts, sucrose, vitamins, BAP, 1-naphthaleneacetic acid (NAA; Sigma-Aldrich), and MES were dissolved in water and the pH was adjusted with KOH or HCl. Then the medium was combined with gellan gum and sterilized by autoclaving at 120°C for 20 min, or alternatively, by filtration through a 0.22-μm PES PLUS membrane (Asahi Glass, Tokyo, Japan) or 0.45-μm PVDF membrane (Miltex HV; Merck-Millipore, Billerica, USA). Hygromycin B, cefotaxime, D-glucose, and acetosyringone were added after autoclaving. Cultivation media with minor modifications, for example those with different concentrations of phytohormones, were prepared in a similar manner.

**Sterilization of seeds**

*M. pudica* “WASE (an early flowering accession)” seeds were purchased from Sakata Seed (Yokohama, Japan). Approximately 400 seeds in a 30-ml conical tube were washed briefly in 20 ml of 70% ethanol, and put under vacuum (-0.8 MPa) for 10 min in another 20 ml of 70% ethanol. Then the seeds were transferred to 20 ml of 50% commercial bleach (TOPVALU Kitchen Bleach; Aeon, Chiba, Japan) containing NaClO, NaOH, and alkylamine-oxide, at concentrations not disclosed by the company, put under vacuum for 10 min, and then washed in another 20 ml of 30% bleach for 30 min with reciprocal shaking at 120 rpm. The seeds were rinsed with sterilized hot water (60°C) at least five times and soaked in 18 ml of hot water (60°C) for 10 min to remove seed coat waxes. After the addition of 2 ml of Plant Preservative Mixture (PPM; Plant Cell Technology, Washington DC, USA), the seeds were put under vacuum for 10 min and then shaken reciprocally at 120 rpm for 30 min. The seeds were placed in a 6×6 array in a Plant Box (a plastic cultivation box with dimensions of 60×60×100 mm; Asahi Glass, Tokyo, Japan) containing 80 ml of GM, and germinated at 25°C for 54 to 60 hours in the dark. Seedlings with hypocotyls of 3 to 8 mm in length were used for subsequent experiments.

**Preparation of explants and optimization of shoot induction conditions**

Explants were prepared under a dissecting microscope in a laminar flow cabinet. Seedlings were dissected on three sheets of filter paper wetted with COM in a petri dish. After the seed coat was removed with forceps, the primary root and cotyledons were
separated from the remaining part of the seedling using a surgical blade (No. 11; Feather, Osaka, Japan) (Figure 1). The epicotyl was cut off from the remaining part to produce the cotyledonary node explant and the associated hypocotyl. Sixteen explants were placed on SIM (25 ml in a 90×20 mm dish) or its derivatives containing different phytohormone concentrations. The explants were cultured at 25°C under 12-hour light (12L; with a light intensity of 120–180 µmol m⁻² s⁻¹)/12-hour dark (12D) cycles, and the medium was changed every 2 weeks. The number of shoots equal to or longer than 2 mm was counted on each explant after 4 or 6 weeks of cultivation.

Transformation

An aliquot of the Agrobacterium stocks was streaked on solid LB medium containing 50 µg/ml hygromycin B and cultured at 30°C for 48 to 60 hours. A single colony was inoculated into 5 ml of liquid LB medium containing 25 µg/ml hygromycin B and precultured at 28°C for 24 hours with rotatory shaking at 180 rpm. After large aggregates were removed by gravity settling, the liquid Agrobacterium culture was again inoculated into 40 ml of fresh liquid LB medium containing 25 µg/ml hygromycin B in a 200 ml baffled flask at a concentration of OD₆₀₀ = 0.15. Then the culture was incubated at 28°C with rotatory shaking at 180 rpm for around 4 hours, until OD₆₀₀ reached 0.6. The Agrobacterium cells were harvested by centrifugation at 5,000×g for 10 min at 25°C, resuspended in 20 ml of COM, centrifuged again, and resuspended in COM at a concentration of OD₆₀₀ = 0.3. Finally, the Agrobacterium suspension was supplemented with 40 µg/ml acetosyringone, 0.2% (w/v) D-glucose, and, in some cases, 0.03% (v/v) Silwet L-77.

Twenty of the cotyledonary node explants were soaked in 10 ml of the Agrobacterium suspension with (or without) 0.03% Silwet L-77 in a glass test tube (16.5×165 mm). In some cases, the explants were sonicated with a Branson Sonifier 150 (Branson Ultrasonics, Danbury, USA) with three pulses of 5-s duration at the maximum output power (14 W). The explants were maintained under normal pressure or vacuum (~0.3 MPa) for 10 min and then collected with a tea strainer. The explants were transferred to a plastic dish (90×15 mm) containing 10 ml of the Agrobacterium suspension without Silwet L-77. Alternatively, the explants were directly transferred to the plastic dish without undergoing sonication, vacuum, or Silwet L-77 treatment. Then the dish was sealed with Parafilm (Bemis, Neenah, USA) and cultured for 3 days at 25°C in the dark. To monitor pH changes in co-cultivation medium, 200 µl of the medium was sampled at each time point and the pH was measured using a compact pH meter (Twin pH AS-212; As One, Osaka, Japan). After the co-cultivation, the explants were transferred to SEM (25 ml in a 90×15 mm dish) with forceps and

Figure 1. Shoot formation from M. pudica explants. A–E. Preparation of explants. A 2-day-old seedling cultured in the dark (A) was divided into the root, the cotyledons with petiole (B), and the remaining part (C). The epicotyl containing the shoot apex was then removed from the remaining part (C) to prepare the cotyledonary node explant (D) as illustrated in (E). Dashed lines in (A), (C), and (E) indicate the cutting positions. The circle in (D) indicates the position of the cotyledonary node. SA, shoot apex; Hc, hypocotyl. F, G. Shoot formation from the cotyledonary node (F) and petiolate cotyledon (G) explants after 4 and 6 weeks of cultivation in the presence of 0.5 mg/ml BAP, respectively. H. Comparison of the frequency of explants forming shoots after 4 weeks of cultivation with 0.5 mg/ml of BAP (n = 32). I, J. Effects of BAP and NAA on shoot formation from cotyledonary node (I) and petiolate cotyledon (J) explants after 4 and 6 weeks of cultivation, respectively. The distribution of the number of shoots formed per explant is shown as box-and-whisker plots (n = 32). Lower and upper whiskers indicate the range of values within 1.5 times the interquartile range from the box and circles indicate outliers. Significant differences were observed between two groups that do not share the same lowercase letter [P < 0.05 by Fisher’s exact test with Holm’s P-value adjustment (H) or Steel-Dwass test (I, J)]. Scale bars, 1 cm (A, F, G), 1 mm (B–D).

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continued to be cultured at 25°C under 12L (with a light intensity of 120–180 μmol m⁻² s⁻¹)/12D cycles, with the medium changed every 5 days. After 10 days of selection on SEM, GFP-positive signals located at the cotyledonary node region of each explant (Figure 1D) were counted visually under a fluorescence dissecting microscope SZX16 (Olympus, Tokyo, Japan) equipped with a SZX2-FGPHQ filter. The number of GFP-positive signals was based on the number of spatially discrete spots, which were predominantly attributable to individual GFP-expressing cells but also included small clusters of cells. The number of explants possessing GFP-positive shoot buds was similarly counted after 30 days of selection. In this experiment, the shoot buds entirely consisting of GFP-positive cells were counted as GFP-positive buds, while chimeric buds containing only some GFP-positive cells were excluded.

Each explant was further cultured on SEM until a GFP-positive callus grew up to 2 mm in length. The GFP-positive callus was surgically excised from the explant and trimmed from GFP-negative tissue. The excised callus was cultured on SEM for an additional 5 days and then cultured on SIM (25 ml in a 90×20 mm dish), with the medium changed every 5 days. After the initiation of shoot elongation, the callus was transferred to a Plant Box containing 80 ml of SIM and continued to be cultured, with the medium changed every 10 days, until the shoots developed at least two compound leaves.

Root induction and whole plant formation

Vermiculite (Fujimi Engei, Shizuoka, Japan) was poured into a Plant Box to a depth of approximately 3 cm, sterilized by autoclaving, and then irrigated with sterilized water, cultivation medium, or phytohormone solution. Cultivation media and water solidified with 0.3% and 1.5% Phytagel, respectively, were also included small clusters of cells. The number of explants possessing GFP-positive shoot buds was similarly counted after 30 days of selection on SEM, GFP-positive buds, while chimeric buds containing only some GFP-positive cells were excluded.

We observed shoot formation from several explants derived from 2-day-old M. pudica seedlings (Figure 1). After a 4-week cultivation in the presence of 0.5 μg/ml 6-benzylaminopurine (BAP), 97% (31 of 32) of the hypocotyls associated with the cotyledonary node (Figure 1D), hereafter referred to as cotyledonary node explants, formed shoots around the node (Figure 1F, H). Lower frequencies of shoot formation were observed from isolated cotyledons with petioles (5 of 32) and hypocotyls cut just beneath the node (10 of 32), and no shoot formation occurred from the basal halves of hypocotyls, roots, or cotyledons that lacked petioles (Figure 1H). These results indicate that tissues in and around the cotyledonary nodes of M. pudica have the ability to form shoots, as do those of other leguminous species [30,31,32].

We optimized the concentrations of two kinds of phytohormones, the cytokinin BAP and the auxin 1-naphthaleneacetic acid (NAA), both of which affect the number of shoots formed on the cotyledonary node in other leguminous species [41,42,43]. Shoots were most efficiently induced on the cotyledonary node in the presence of 0.5 μg/ml BAP and no NAA, which resulted in 5.2±0.5 (mean ± SE) shoots per explant after 4 weeks of cultivation (n = 32; Figure 1I). Shoot formation frequency of petiolate cotyledon explants was also examined at various phytohormone concentrations, but only 2.1±0.4 shoots per explant or fewer were induced, even after a longer cultivation period (n = 32; 6 weeks; Figure 1J). Based on these observations, the cotyledonary node was selected as the target tissue for...
Agrobacterium infection, and explants were cultured in medium supplemented with 0.5 μg/ml BAP in subsequent experiments.

**Agrobacterium-mediated transformation of cotyledonal nodes**

For the Agrobacterium-mediated transformation of cotyledonal node cells, we prepared two kinds of binary vectors: a conventional binary vector, pIF121-Hm, and a super-binary vector, pSB111-GFP, which possesses additional virulence genes [33,34]. The T-DNA region of each vector carries an intron-sGFP reporter gene (Figure 2A), which can be used to selectively visualize transformed cells in living plant tissues, but does not label Agrobacterium cells [37].

*Agrobacterium tumefaciens* infection is triggered by the transcriptional activation of its virulence genes [44] in response to phenolic compounds such as acetosyringone [45], monosaccharides [46,47], and acidic pH values [48]. We thus examined the effects of supplementing the co-cultivation medium with acetosyringone, D-glucose, and MES buffer adjusted to pH 5.8, alone or in combination, on transformation efficiency (Figure 2B). The number of GFP-positive signals in the cotyledonal node increased in the presence of both acetosyringone and MES (Figure 2B). Although the addition of glucose further increased the transformation efficiency of explants treated with acetosyringone and MES buffer, the increase was not significant (Figure 2B). However, since the addition of glucose did potentially increase the transformation efficiency, we used all three compounds in the subsequent experiments. A comparison of the two binary vectors and Agrobacterium strains demonstrated that the transformation efficiency was higher when pSB111-GFP was combined with Agrobacterium strain LBA4404 than when pIF121-Hm was combined with any of the four different Agrobacterium strains (Figure 2C). These results suggest that the addition of acetosyringone and of a buffer capable of maintaining an acidic pH enhance the transformation efficiency of *M. pudica*, as does the use of a super-binary vector.

We further assessed the effect of pH on Agrobacterium infection. As reported previously [49], the addition of MES to cultivation media reduced the amount of pH changes during autoclaving (Figure 5A). However, the smaller change in pH after autoclaving was not the direct cause of the improved infection efficiency, because the cotyledonal nodes of explants cultured on filtration-sterilized, non-buffered medium had almost no positive signals, as did those cultured on autoclaved, non-buffered medium (Figure 5B). Monitoring the pH of the medium during the co-cultivation period revealed that the pH in the non-buffered medium containing Agrobacterium and the explants dropped below 4.7 within the first three hours (Figure 3A). A similar decrease in pH was observed in the non-buffered medium containing only Agrobacterium, but not in the medium alone or in medium containing only explants (Figure 3A), suggesting that the conspicuous acidification of the co-cultivation medium was mainly caused by Agrobacterium. The addition of MES buffer relieved, but did not completely prevent, the excessive acidification and kept the pH of the medium above 5.0 for at least 9 hours during co-cultivation (Figure 3A). The addition of MES also improved the transformation efficiency when using a solid co-cultivation medium, but to a lesser extent than the liquid co-cultivation medium (Figure 5C). Optimization of the initial pH value demonstrated that transformation was most efficient in liquid co-cultivation medium adjusted to pH 6.1 (Figure 2D). This value was higher than those reported for the maximum induction of virulence genes in octopine-type Agrobacterium strains (pH 5.2 to 5.3) [48,50], and possibly counterbalanced the pH decrease during co-cultivation (Figure 3B).

To further improve the transformation efficiency, we examined the effects of sonication [51] and vacuum infiltration [52] prior to the co-cultivation period. We also gauged the effects of transiently adding a detergent, Silwet L-77 [53,54], to the Agrobacterium suspension at 0.05% (v/v) during the sonication and/or vacuum treatments. Compared to the control experiment, none of the treatments, individually or combined, significantly altered the number of GFP-positive signals after 10 days of selection (Figure 2E). On the other hand, the number of explants forming GFP-positive shoot buds after 30 days varied depending on the treatments (Figure 2F). A significant, two-fold increase was observed in the explants treated only with Silwet L-77 (Figure 2F), suggesting that the detergent facilitates Agrobacterium infection of cells that are capable of forming shoots, which are possibly situated deep inside the cotyledonal node. The additional use of sonication and/or vacuum in combination with the Silwet L-77 treatment reduced the emergence of GFP-positive shoot buds (Figure 2F), possibly due to the increased damage of cells at the cotyledonal node.

Taken together, the transformation efficiency of the cotyledonal node of *M. pudica* was drastically improved by three different factors: the use of the super-binary vector, the addition of MES buffer to the co-cultivation medium, and transient treatment with Silwet L-77 before co-cultivation.

**Root induction and whole plant formation**

After 1 month or longer of selection with hygromycin B, transformed cells in the cotyledonal node formed GFP-positive calluses with shoot buds (Figure 2F). These calluses were surgically isolated from the explants and continued to be cultured on SIM for further shoot development (Figure 2K-M). Well-developed shoots possessing at least two compound leaves (Figure 4A, B) were used in a root induction experiment in which three nutrient conditions (water, 1/2 MS, or 1/2 MS containing sucrose and vitamins) and two supporting materials (gellan gum or vermiculite) were tested. For both supporting materials, higher root induction efficiencies were obtained with water than with the MS-based media (Figure 4E), suggesting that poor nutrient conditions favored rooting. Vermiculite increased root induction efficiencies to a greater extent than did gellan gum (Figure 4E), possibly due to the improved permeability to air [55]. Roots were most efficiently induced by vermiculite supplied with water, which resulted in rooting of 81% (22 of 27) of the regenerated shoots after 21 days of cultivation (Figure 4E). This efficiency, together with the fact that the transformed shoots can readily be multiplied by vegetative propagation on SIM, ensures the root induction on practically all transformed shoots. We also examined the effects of three auxins, NAA, indole-3-acetic acid (IAA), and indole-3-butyric acid (IBA), all of which were used for root induction in various plants [43,56,57,58]. None of these compounds, however, improved the root induction efficiency of our system any further, when used at a concentration of 0.5 μg/ml (Figure 4E). The resultant plantlets were transferred to soil after their roots reached 5 cm in total length (Figure 4C, D) and their establishment in the soil was confirmed by further cultivation.

Using the optimized conditions described above, we evaluated the transformation efficiency of *M. pudica* throughout the entire procedure. A total of 160 cotyledonal node explants were subjected to the Agrobacterium-mediated transformation, and monitored for 12 months after co-cultivation (Figure 5A). Sixty-three percent (101 of 160) of the explants formed GFP-positive calluses during selection and more than a half of them (57 of 101)
initiated shoot elongation on SIM. Forty-two of the 57 shoots developed two or more compound leaves and 30 of these successfully rooted and became established in the soil. These results demonstrated that 18.8% of the explants (30 of 160) produced at least one independent line of T₀ plants (Figure 5A).

The number of transgenic T₀ plants continued to increase even after 12 months of cultivation (Figure 5A), suggesting that the efficiency would further increase with time. On the other hand, four independent T₀ plants (derived from 2.5% of the explants) became established in as little as 4 months (Figure 5A), enabling us to recover their T₁ progeny within a total of 8 months (Figure 5B).

Molecular and biological analyses of transgenic plants

We performed a genomic Southern blot analysis on regenerated T₀ plants using the sgfp sequence as a probe (Figure 2A). Among 15 independent lines tested, approximately two-thirds of transgenic plants (9 of 15) possessed a single T-DNA insertion, while the others (4 of 15) had two insertions (Figure 6). This simple pattern of insertion represents an advantage of the present method over particle bombardment, which is used to transform other leguminous species [30,31], but which often results in complex patterns of DNA insertions [59,60].

Transmission of the transgene to T₁ progeny was confirmed in all lines tested (n = 10) by observing the GFP fluorescence (Figure 5C). In most cases (9 of 10), the segregation ratio of the GFP fluorescence in a selfed T₁ progeny was in good agreement with that expected from the number of T-DNA insertions (Table S1; 3:1 and 15:1 for one and two T-DNA insertions, respectively). These results provide further evidence for the simplicity of T-DNA insertion patterns produced by the present method and also indicate the non-chimeric nature of each T₀ plant. Transmission of the transgene to T₂ progeny was also confirmed for one line (Figure 5D), demonstrating the stable transmission of transgenes across generations.

Finally, the transgenic plants were examined for their ability to undergo characteristic movements. All of the T₀ (n = 70) and T₁ (n = 10) plants showed both seismonastic movement in response to touch (Video S1) and nictinastic movement (data not shown), suggesting that the transformation procedure presented here does not impair these movements. In sum, the present study provides a genetic tool to investigate the molecular mechanisms underlying the intriguing movements of M. pudica.

Discussion

In this study, we developed a robust protocol for the genetic transformation of M. pudica. A key improvement for the successful transformation of M. pudica was the use of MES buffer to maintain...
the pH during co-cultivation. Although the pH-dependent activation of Agrobacterium virulence genes was previously demonstrated [48,50] and several studies emphasized the importance of buffering agents in co-cultivation media [61,62], the requirement to stabilize the pH with buffering agents seems to depend on the transformation system being used. For example, only one-quarter (17 of 67) of the transformation methods given in a protocol book [27] that covers a wide range of plant species and transformation systems describes the use of buffer reagents during Agrobacterium preparation and/or co-cultivation. This variability may be due to differences in other conditions that possibly affected pH stability, such as the composition of co-cultivation media, the
In this study, the transgenic plants were recovered via a combination of hygromycin selection and fluorescence-based visual selection, in which GFP-positive transgenic calluses were surgically isolated from surrounding non-transgenic regions. Compared to hygromycin selection alone, this dual selection system facilitates and accelerates the establishment of transgenic T₀ plants that consist entirely of transformed cells. On the other hand, our preliminary observation indicated that 70% (21 of 30) of the regenerated shoots (>5 mm) exhibited GFP fluorescence after 60 days of cultivation on SEM. This result suggests that transformatnts can also be recovered using antibiotic selection alone, although further investigation is required to evaluate the recovery rate of transgenic plants under these conditions.

Despite the recent development of new reverse genetic tools, such as virus-induced gene silencing (VIGS) [71], Agrobacterium-mediated transformation still plays a pivotal role in plant biology research. In the present study, we establish a method whereby this invaluable genetic technique may be applied to *M. pudica*, a classic model organism in plant physiology.

### Supporting Information

**Figure S1 Effects of MES buffer on transformation efficiency.** A. Changes in pH of co-cultivation media after autoclaving. Data are the means ± SD (n = 5). A diagonal line is shown for clear visualization of the pH changes from initial values. B. Comparison of sterilization methods of co-cultivation media in the presence or absence of 0.1% MES buffer (n = 20). Each co-cultivation medium was adjusted to pH 5.8 before sterilization and supplemented with both acetosyringone and glucose. C. Comparison of liquid and solid co-cultivation media in the presence or absence of 0.1% MES buffer (n = 20). The pH of each co-cultivation medium was adjusted to 3.8 before autoclaving. Gellan gum (0.3%) was used to solidify the solid co-cultivation media. The number of GFP-positive signals on the cotyledonary node of each explant was counted after 10 days of selection. Significant differences (P<0.05 by the Steel-Dwass test) were observed between two groups that do not share the same lowercase letter (B, C). D, E. Comparison of sterilization methods of co-cultivation media in the presence or absence of 0.1% MES buffer (n = 20). Each co-cultivation medium optimized for transformation (n = 80). Co-cultivation medium containing acetosyringone, glucose, and MES buffer (pH 6.1) and the treatment with Silwet L-77 prior to co-cultivation were used in this experiment. No significant differences were observed in either the number of GFP-positive signals after 10 days of selection (D) by the Mann-Whitney U-test or the frequency of explants possessing GFP-positive shoot buds after 30 days of selection (E; by Fisher’s exact test).

(TIF)

**Table S1 Segregation of GFP expression in selfed T₁ progeny.**

(TIF)

**Video S1 Movie of the seismonastic movement of transgenic *M. pudica*.** A homozygous T₁ seedling (10 days old) is shown. Green and red signals represent the GFP fluorescence and the autofluorescence of chloroplasts, respectively. This movie was taken using a SZX16 microscope equipped with a SZX2-FGFP long-pass filter and coupled to a DP71 digital camera (Olympus).

(WMV)

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Author Contributions
Conceived and designed the experiments: HM YH MH. Performed the experiments: HM TF NS. Analyzed the data: HM. Wrote the paper: HM YH MH.

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