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**Note**

# A simple method for *in planta* tomato transformation by inoculating floral buds with a sticky *Agrobacterium tumefaciens* suspension

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**Abstract** Tomato transformation is conventionally performed using *Agrobacterium tumefaciens*-infected cotyledons. Here, we propose a simple procedure for tomato transformation, by which *A. tumefaciens* cells were smeared onto floral buds of a tomato plant using a paintbrush. Sufficient numbers of fruits were obtained from them, although the smearing of an excess number of *A. tumefaciens* cells led to an adverse effect on the plant growth. Progeny plants were screened by growth on a kanamycin-containing selection medium plate. The *nptII* gene was detected in 10 plants among 1,599 progenies. These transformants were derived from fruits other than those obtained from the smeared buds. This suggested that *A. tumefaciens* cells moved to the buds located near the smeared buds and caused the transformation event. Our findings suggest that this procedure can be used for the introduction of a foreign gene into plant cells.

**Key words:** *Agrobacterium*-mediated transformation, floral dip with smearing method, kanamycin resistance, mature tomato plant.

Tomato transformation is generally performed using a method involving cotyledons of seedlings, in which cultured tissues obtained from the cotyledons are infected with *Agrobacterium tumefaciens* and regenerated on a selection medium (McCormick et al. 1986). This method often requires time and skilled labor to obtain a transformant line (Ling et al. 1998; van Roekel et al. 1993). Currently, transformation of *Arabidopsis thaliana* is performed using the floral dip method. For this method, *A. tumefaciens* cells harboring an appropriate gene are infected into an immature flower bud, by which the desired gene is introduced into the plant cells. Usually, transformant plants are obtained among plants from the resulting seeds (Clough and Bent 1998). In this study, we attempted to obtain a transformant tomato plant using a floral smearing method and established this method as a simple procedure for tomato transformation.

To determine the adequate number of *A. tumefaciens* cells for smearing on the floral buds, we investigated the transformation efficiency when a series of bacterial concentrations was used. For the leaf-disk method for tomato transformation, it has been reported that the concentration of *A. tumefaciens* cells may strongly affect the transformation efficiency, and that the highest efficiency was obtained when a cell suspension with $OD_{600}=0.2$ was used (Pawar et al. 2013). In this study, we determined the influence of concentration of *A. tumefaciens* cells on the floral dip treatment.

*A. tumefaciens* GV3101 strain harboring pBI121 vector was cultured in a liquid medium, and bacterial cells were harvested. They were suspended in the tomato infection medium (Murashige and Skoog Plant Salt Mixture (Sigma-Aldrich, St. Louis, MO, U.S.A.) 4.6 g $l^{-1}$, MES 250 mg $l^{-1}$, sucrose 50 g $l^{-1}$, 1 N KOH 800 $\mu$l $l^{-1}$, 6-Benzylaminopurine (BA) $1.0\times10^{-2}$ mg $l^{-1}$, Tween-20 200 $\mu$l $l^{-1}$, acetosyringone 20 mg $l^{-1}$) and used to prepare a series of *A. tumefaciens* suspension cocktails. Cell suspensions with a series of concentrations were adjusted to have $OD_{600}=0.2$, $OD_{600}=0.4$, $OD_{600}=0.6$, and $OD_{600}=0.8$, using the McFarland nephelometric method (Washington et al. 1972), with estimated concentrations of approximately $2.5\times10^4$, $5.0\times10^4$, $7.5\times10^4$, and $10.0\times10^4$ cells $ml^{-1}$, respectively. Then, 1/100 amount of 'Nenchaku-kun' (Sumitomo Chemical Co., Tokyo, Japan), a spreading agent (a kind of pasted starch), was added to the cell suspensions. The resultant sticky *A. tumefaciens*

Abbreviations: *nptII*, the gene for kanamycin resistance; $OD_{600}$, optical density at 600 nm.
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suspension cocktails were smeared using a paintbrush on floral buds of Micro-Tom tomato, which was cultivated for 1 month after sowing. Each *A. tumefaciens* suspension cocktail was applied to three individual plants and smeared on five or six immature buds of each plant (Figure 1). After this treatment, tomato plants were put into a plastic container and decompressed for 10 min. Then, they were covered with a plastic wrapping film and incubated for 24 h in a growth chamber at 22°C under the long-day condition (16 h in light and 8 h in dark). After removal of the wrapping film, they were subsequently cultured in the growth cabinet under the same light condition.

Sufficient numbers of fruits were obtained from the tomato plants treated with the *A. tumefaciens* suspensions with $OD_{600}$=0.2, $OD_{600}$=0.4, and $OD_{600}$=0.6, but few fruits were generated from that with $OD_{600}$=0.8 because of growth inhibition (Table 1). These findings suggested that an excess amount of *A. tumefaciens* resulted in an adverse effect on the plant growth.

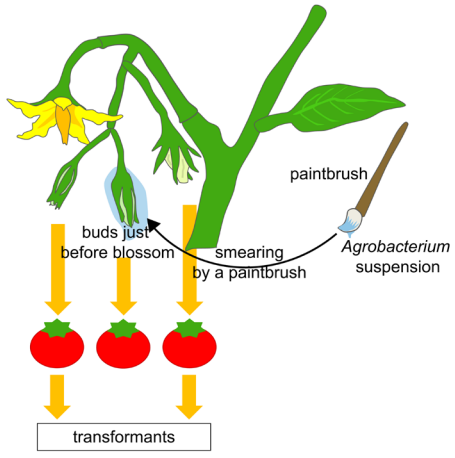


Figure 1. Schematic representation for the procedure of *Agrobacterium tumefaciens* smearing onto tomato buds. A suspension of *A. tumefaciens* cells combined with a spreading agent was smeared using a paintbrush onto the buds just before they blossomed.

Seeds were harvested from fruits derived from the smeared buds and those located near these buds. The seeds of each plant were sown on a kanamycin-containing selection medium plate and germinated. Seedlings were cultivated for 4 weeks at 22°C under the long-day condition (16 h in light and 8 h in dark).

Usually, kanamycin-resistant tomato plants are selected by generation and elongation of a lateral root from the major root of the plants that are grown on a medium containing kanamycin. Although we attempted to select kanamycin-resistant plants in the same manner, it was rather difficult to detect the presence of kanamycin resistance because most of the plants produced a lateral root but only limited elongation occurred in 2 weeks of culturing. Therefore, we continued to culture them for at least 1 month and selected the well-grown plants (Table 1). In this way, we succeeded in the first screening and obtained a pool containing well-grown transformant plants.

Although many plants soon stopped growing, some plants showed generation of more than three branched roots with green leaves, suggesting that they had survived on the kanamycin-containing medium. The proportions of these plants in those obtained from germinated seeds of plants treated with *A. tumefaciens* suspensions with $OD_{600}$=0.2, $OD_{600}$=0.4, and $OD_{600}$=0.6 were 11.6%, 3.7%, and 7.2%, respectively. In total, we obtained 113 plants out of 1,599 progeny seeds from eight plants treated with the *A. tumefaciens* suspensions (Table 2).

Next, we attempted to detect the kanamycin resistance gene *nptII*, derived from pBI121. Genomic DNA was prepared from leaves of the plants selected on the kanamycin-containing medium and used for analysis of the presence of *nptII*. Among the selected plants, we detected that some plants showed amplification

Table 1. Number of fruits from the plants inoculated with *Agrobacterium tumefaciens* suspension.

| Experimental ID | Number of buds with treatment | Number of total fruits | Number of fruits from the buds with treatment | Number of fruits from the buds without treatment |
|---|---|---|---|---|
| $OD_{600}$=0.2 #1 | 5 | 26 | 1 | 25 |
| $OD_{600}$=0.2 #2 | 5 | 25 | 1 | 24 |
| $OD_{600}$=0.2 #3 | 5 | 26 | 2 | 24 |
| $OD_{600}$=0.4 #1 | 5 | 22 | 0 | 22 |
| $OD_{600}$=0.4 #2 | 6 | 26 | 0 | 26 |
| $OD_{600}$=0.4 #3 | 5 | 22 | 0 | 22 |
| $OD_{600}$=0.6 #1 | 6 | 25 | 0 | 25 |
| $OD_{600}$=0.6 #2 | 5 | 29 | 3 | 26 |
| $OD_{600}$=0.6 #3 | 6 | 25 | 0 | 25 |
| $OD_{600}$=0.8 #1 | 5 | 13 | 0 | 13 |
| $OD_{600}$=0.8 #2 | 5 | 8 | 0 | 8 |
| $OD_{600}$=0.8 #3 | 5 | 3 | 0 | 3 |

Inoculation of each *A. tumefaciens* cell suspension with a different concentration was performed on the buds of three individual plants. Experimental ID indicates the name of tomato plant corresponding to each experiment. Each experiment using a series of concentrations of *A. tumefaciens* cell suspension was performed in triplicate.

Table 2. Number of seeds from fruits obtained from the plants smeared with *Agrobacterium tumefaciens* suspensions.

| Experimental ID | Number of total seeds harvested | Number of seeds germinated (A) | Number of grown plants | Number of plants in which the *nptII* gene was detected (B) | Ratio of (B)/(A) (%) |
|---|---|---|---|---|---|
| $OD_{600}$=0.2 #1 | 253 | 210 | 26 | 2 | 0.95 |
| $OD_{600}$=0.2 #2 | 219 | 189 | 18 | 0 | 0 |
| $OD_{600}$=0.2 #3 | 39 | 30 | 6 | 0 | 0 |
| Subtotal | 511 | 429 | 50 | 2 | 0.47 |
| $OD_{600}$=0.4 #1 | 201 | 164 | 5 | 0 | 0 |
| $OD_{600}$=0.4 #2 | 298 | 225 | 3 | 0 | 0 |
| $OD_{600}$=0.4 #3 | 196 | 155 | 12 | 1 | 0.65 |
| Subtotal | 695 | 544 | 20 | 1 | 0.18 |
| $OD_{600}$=0.6 #1 | 49 | 36 | 0 | 0 | 0 |
| $OD_{600}$=0.6 #2 | 336 | 307 | 23 | 0 | 0 |
| $OD_{600}$=0.6 #3 | 275 | 256 | 20 | 7 | 2.73 |
| Subtotal | 660 | 599 | 43 | 7 | 1.17 |
| $OD_{600}$=0.8 #1 | 18 | 12 | 0 | 0 | 0 |
| $OD_{600}$=0.8 #2 | 20 | 15 | 0 | 0 | 0 |
| $OD_{600}$=0.8 #3 | 0 | 0 | 0 | 0 | 0 |
| Subtotal | 38 | 27 | 0 | 0 | 0 |
| Total | 1,904 | 1,599 | 113 | 10 | 0.63 |

Experimental ID indicates the name of tomato plant corresponding to each experiment. Numbers of total seeds harvested from the individual tomato plants, numbers of seeds that germinated among them, numbers of grown plants selected on the kanamycin-containing medium, numbers of plants in which the *nptII* gene was detected, and the ratio of transformants in the germinated seeds are indicated. Subtotals show the total values in the experiments using the *A. tumefaciens* suspensions of the same concentration.

of the specific DNA fragment corresponding to *nptII*, suggesting that they were the transformants containing *nptII* (Figure 2A). In addition, to detect the DNA fragment derived from the remaining *Agrobacterium* cells in the progeny plants, we were subjected to PCR-amplification of the DNA fragment corresponding to the *virG* gene in the Ti plasmid of *A. tumefaciens* using the *Agrobacterium*-specific primers. However, no corresponding fragment was detected in these plants (Figure 2B). These results suggest that they no longer contained *Agrobacterium* cells, and that these plants were transformed by the integration of the kanamycin resistance gene. We obtained two transformant plants derived from the fruits of a plant treated with *A. tumefaciens* suspension with $OD_{600}$=0.2 (named as $OD_{600}$=0.2 #1), one plant derived from a plant treated with the suspension with $OD_{600}$=0.4 ($OD_{600}$=0.4 #3), and seven plants derived from a plant treated with the suspension with $OD_{600}$=0.6 ($OD_{600}$=0.6 #3). This finding also indicated that our procedure could be used for the introduction of a foreign gene into plant cells. When the *A. tumefaciens* suspension with $OD_{600}$=0.6 was smeared on the buds, the highest transformation ratio was achieved. In this case, the ratio of transformants in the germinated seeds was 2.73% (Table 2).

We analyzed transformation frequency for the seeds of fruits derived from buds inoculated with *A. tumefaciens* cell suspension, and those located near these buds. The ratio of transformants obtained from

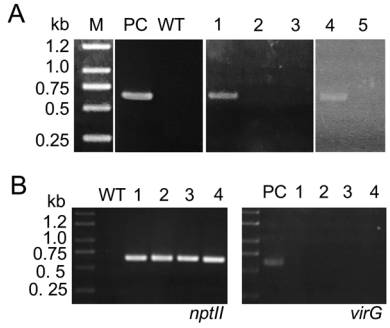


Figure 2. Detection of the transgene. (A) Detection of the *nptII* gene in the transformants. PC, pBI121 DNA; WT, genomic DNA of the wild-type plant; 1 to 5, genomic DNA prepared from representative progenies. 1 to 3 are derived from the progenies of $OD_{600}$=0.2 #1, and 4 and 5 are derived from those of $OD_{600}$=0.6 #3. A specific DNA fragment corresponding to *nptII* was polymerase chain reaction (PCR)-amplified from the genomic DNA using a set of primers, 5′-AATATCACGGGTAGCCAACG-3′ and 5′-GCTTGGGTGGAGAGGCTATT-3′, which was based on the plasmid pBI121 (acc. number: AF485783). (B) Evaluation of the transformants. The existence of *nptII* (left panel) and *virG* (right panel) genes was examined in the transformants. Numbers indicate the progenies derived from the buds of $OD_{600}$=0.6 #3. Each number corresponds to the same individual plant. A specific fragment corresponding to *virG* (acc. number: X04965) was PCR-amplified using a set of primers, 5′-TCGATGACGACGTCGCTATG-3′ and 5′-CGCAGCCTCAAAATGAGAAC-3′, which was based on the Ti plasmid. PC, *Agrobacterium tumefaciens* DNA; WT, genomic DNA of the wild-type plant; 1 to 4, genomic DNA prepared from representative transformants.

Table 3. Number of transformants derived from buds of treated plants.

| Experimental ID | Seeds from smeared buds | | Seeds from other buds | |
|---|---|---|---|---|
| | Number of seeds | Number of transformants | Number of seeds | Number of transformants |
| $OD_{600}$=0.2 #1 | 5 | 0 | 248 | 2 |
| $OD_{600}$=0.4 #3 | 0 | 0 | 196 | 1 |
| $OD_{600}$=0.6 #3 | 0 | 0 | 275 | 7 |

Experimental ID indicates the name of tomato plant that produced transformants containing the *nptII* gene. Numbers of seeds and transformants in the progenies generated by the buds with/without *Agrobacterium tumefaciens* treatment are shown.

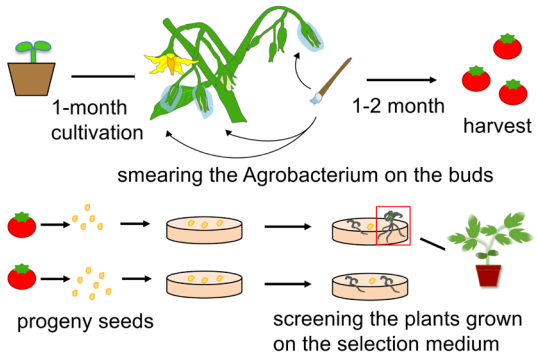


Figure 3. Schematic representation of the proposed procedure of tomato transformation. *Agrobacterium tumefaciens* smearing was performed on the buds of 1-month-old tomato plants after germination. Seeds were harvested from the fruits generated on the treated plants. They were sown on the selection medium containing kanamycin. Among the plants that grew well, transformants were selected.

the buds smeared with *A. tumefaciens* suspension was compared with that for the buds located near them. Some of the smeared buds were falling before ripening, and only a small number of fruits was generated from them. No transformant was obtained among the seeds derived from the fruits derived from these buds. All transformants containing the kanamycin resistance gene were derived from the buds other than the smeared buds (Table 3). This finding suggested that *A. tumefaciens* moved to the buds located near the smeared buds and caused the transformation event.

Transformation of tomato using the floral dip method based on a technique different from the usual procedure has been reported, with a transformation efficiency of 0.25–0.5% for floral dips or floral injections into the buds (Sharada et al. 2017). A procedure for floral dip transformation of *Brassica rapa* has been proposed by Hu et al. (2019). They suggested that efficient transformation occurs when a plant at an immature stage is used. Here, we proposed a simple procedure for tomato transformation by smearing *A. tumefaciens* suspension onto floral buds (Figure 3). The conventional method for tomato transformation has been established (McCormick et al. 1986). Our method exhibited a similar efficiency to the conventional one, and it would be convenient to achieve the tomato transformation because it requires less skilled labor under the clean experimental condition.

Although some buds were falling before ripening and no transformant was obtained from the fruits derived from the smeared buds, we found that the generation of transformants occurred from the seeds derived from buds located near the buds smeared with *A. tumefaciens* cells. Our findings indicated that *A. tumefaciens* moved to other buds on the plant with the smeared buds and led to a transformation event in embryos located in the other buds. Therefore, these findings indicate that this procedure results in a sufficient number of transformants when well-grown plants with multiple flowers are used. In addition, this procedure may be applied to other tomato cultivars and contribute to their molecular breeding.

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