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PRICK AND SOAK *Agroacterium tumefaciens*-MEDIATED *IN PLANTA* TRANSFORMATION IN TOMATO (*Lycopersicon esculentum* Mill.)

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ABSTRACT

One of the modern plant breedings through genetic engineering is *Agrobacterium tumefaciens*-mediated transformation. *Agrobacterium tumefaciens*-mediated transformation can be performed *in vitro* or *in planta*. *In planta* transformation arises from the weaknesses of the *in vitro* method such as need high hygiene standard, professional tissue culture experts, and more time to prepare explants and somaclonal variation. *In planta* transformation is a method to transfer the gene to the plant genome without any tissue culture stages. The aims of this research were to know the possibility of the prick and soak *in planta* method with the target of tomato seeds and to know the most suitable inoculation time for tomato seeds transformation by prick and soak method the transformation is done by pricking the seeds and soaking them in the *A. tumefaciens* suspension. The treatments in this study were 1 and 2 days inoculation time to test the efficacy of prick and soak *in planta* transformation method. Tomato seeds were pricked with a needle on the center once, and then soaked in *A. tumefaciens* strain LB4404 suspension carrying *pKYS-SoSPS*1 plasmid with Neomycin Phosphotransferase (*NPT*II) and *Saccharum officinarum* Sucrose Phosphate synthase (*SoSPS*1) genes. Visualization of tomato's DNA samples after PCR showed that 1-day inoculation sample was positively integrated with *NPT*II gene and negative in the 2 days inoculation treatment.

Keywords: Tomato seeds, *In planta* Transformation, *Agrobacterium tumefaciens*, prick and soak

INTRODUCTION

Tomato is one of the many cultivated plants in Indonesia used as processed materials and fresh consumption. The need for tomatoes requires not only high production but also good quality. Good quality of tomato can be achieved by making tomato plants varieties with superior properties such as high production, response to fertilization, high vitamin content, sweet taste, fast fruiting, resistant to biotic and abiotic stresses and many more. Tomato plants varieties with superior properties can be obtained by conventional cross-breeding or genetic engineering. Genetic engineering is widely done by scientist lately. Despite its low success rate (Pardal, 2002, Utomo, 2004), genetic engineering is relatively fast (Dwiyani *et al*., 2016a), can be done with standard laboratory types of equipment

(Rahmawati, 2006) and does not require spacious place compared to conventional cross-breeding (Azrai, 2005).

Genetic engineering in plants can be done by microprojectile bombardment, electroporation, silicon carbide-mediated transformation, and *Agrobacterium tumefaciens*-mediated transformation (Dwiyani *et al*., 2016a). *A. tumefaciens*-mediated transformation is one of the most widely developed genetic engineering methods. *A. tumefaciens*-mediated transformation method is relatively easy to do, can be done with standard laboratory types of equipment, cheaper cost and plant genome can be derived to the progenies compared to the other genetic engineering methods (Aldemita and Hodges, 1996).

A. tumefaciens-mediated transformation can be done *in vitro* and *in planta*. *In vitro* transformation uses tissue culture stages for transfer gene process, so it requires sterile conditions during the transformation process. In addition, *in vitro* transformation has several disadvantages including the needs for tissue culture experts, a long time in the provision of explants and sometimes produces somaclonal variation plants (Ping *et al*., 2003). From the weaknesses in *in vitro* transformation, the researchers turned to transformation without using any tissue culture stages. The transformation method performed directly without going through the tissue culture stages is defined as *in planta* transformation by Feldman and Mark (1987).

In planta transformation has been performed in several plants such as *Arabidopsis thaliana* (Clough and Bent, 1998; Bent. 2000; Barik, 2013; Narusaka *et al*., 2010; Zhang *et al*., 2006), *Brassica napus* (Li *et al*., 2010), orchids (Semiarti, 2012), *Kalanchoe* sp. (Dewanto and Suhandono, 2016), wheat (Razzaq *et al*., 2011), rice (Rod-in *et al*., 2014; Ratanasut *et al*., 2017), soybean (Shou *et al*., 2002; Zia *et al*., 2011) and tomatoes (Hasan *et al*., 2008). The floral dip is the most frequent *in planta* transformation method performed in tomato plants. Floral dip method uses flowers as the target of transformation.

Floral dip *in planta* transformation stages relatively takes a long time. After transforming the flowers, we still have to wait for the flowers to become ripe fruits, and then the seeds from the fruits grown to be selected as candidate transformants. The idea arises to cut the time of the research by performing a direct transformation on the tomato seed. Until now, there has been no report on the use of tomato seeds directly in *in planta* transformation. In addition, to shorten the time, the target of the transformation of seeds is also easier to obtain because it is always available in agricultural shops.

This research used *in planta* transformation method with the target of

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tomato seeds. The seeds are treated by pricking and then soaking in the *A. tumefaciens* suspension so that this method is called the prick and soak method. This method has not been reported by other researchers so the result of this study has novelty value.

According to Dwiyani *et al*. (2016b) inoculation time becomes an important factor in transformation. This study used soak time as the treatment. Soaking is done for as long as possible with the consideration that the longer the inoculation time, the longer the chances of *A. tumefaciens* in transferring the gene to the embryo in the seeds. To determine the treatment in this research, there was preliminary research by soaking the pricked seeds for 1 day and 2 days using water. The results showed that the seeds can still germinate after soaked for 2 days with the number of seeds germinate as much as 22%. So it was decided for the treatment in this study is soaking the pricked tomato seeds for 1 and 2 days, because if the seeds soaked longer, it is feared it will make the seeds rotted.

MATERIALS AND METHODS

Confirmation of *NPT*II gene in *A. tumefaciens* via PCR (polymerase chain reaction) and electrophoresis

The bacteria were strike on a solid LB (Luria Bertani) medium with 50 ppm kanamycin, 100 ppm rifampicin and 12.5 ppm gentamicin antibiotics, incubated in 25ºC for 48 hours. Ten single colonies of *A. tumefaciens* were taken for amplification on PCR and struck on different points in solid LB medium and marked.Samples were amplified in PCR machine with *NPT*II (neomycin phosphotransferase) primer (forward: 5’-GTCATCTCACCTTCCTCCTGCC 3’; reverse: 5’GTCGCTTGGTCGGTCATTTCG-3’), *NPT*II is a kanamycin resistance gene which is the selectable marker in T-DNA construct of *the pKYS-SoSPS*1 plasmid. *NPT*II PCR program with predenaturation 95ºC for 3 minutes, denaturation 95ºC for 30 seconds, annealing 59ºC 30 for seconds, extension 72ºC for 1 minute, final extension 72ºC for 5 minutes (Manders *et al*., 1994). The PCR cycle was performed 35 times with expected target of 550 bp DNA amplified. The results of PCR were electrophoresed then the results were visualized. Positive *A. tumefaciens* samples containing the *NPT*II gene were used for transformation.

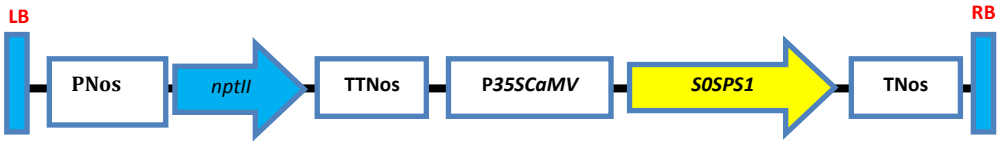

Fig. 1.Construct of T-DNA in *pKYS-SoSPS*1 plasmid by Sugiharto *et al.* (1997).
T-DNA in *pKYS-SoSPS*1 plasmid contain *left border* (LB), *promoter nopaline synthase* (PNos), *neomycin phosphotransferase* (*NPT*II), *terminator nopaline synthase* (TNos), *promoter CaMV* (P35S*CaMV*), *Saccharum officinarum sucrose phosphate synthase* (*SoSPS*1), *terminator nopaline synthase* (TNos) and *right border* (RB) as the border of T-DNA

Preparation of *A. tumefaciens* suspension

PCR positive of single colonies of *A. tumefaciens* were grown on 2 ml LB medium with 50 ppm kanamycin, 100 ppm rifampicin and 12.5 ppm gentamicin antibiotics then incubated at 150 rpm shaker at 28° C for 24 hours until OD reaches 0.6. Every 1 ml of *A. tumefaciens* culture was subcultured to 20 ml LB and added with as previous antibiotics concentration then incubated at 150 rpm shaker at 28 ° C for 24 hours until OD reached 0.6. The bacteria are then transferred to the test tubes with a size of 10 ml each tube. The tube is closed and centrifuged at 5,000 rpm for 10 minutes. The supernatant was removed and the precipitate suspended on ½ NP medium containing 100 ppm acetosyringone and 30 µl tween20 until the volume reached 20 ml (Dewanti *et al.*, 2011).

Prick and soak seed transformation

200 tomato seeds were pricked with a needle. Tomato seeds are pricked in the center and then soaked in *A. tumefaciens* suspension prepared before. 100 seeds were soaked for one day and the remaining 100 seeds were soaked for two days. The seeds are then filtered and planted in wet tissue on the petri dish to germinate for nine days and then the sprouts transferred in soil medium.

Percentage of seeds that grew after the transformation

After prick and soak transformation treatment, the germinated seeds were percentage (GP) with the formula:

$$GP = \frac{germinated\ seeds}{Total\ seeds\ transformed}\ x\ 100\%$$

Isolation of tomato plant DNA and electrophoresis

Young leaves of the Tomato plants were taken about 3 leaves per plant. The leaves then were put into the plastic and labeled and then stored in ice box containing ice packs. Tomato plant DNA was isolated by a modification of Mini Prep-Method by Zang and Stewart (2000). Modifications include the use of fewer samples and manual grinding method with just a drill bit without the electric drill. The isolated DNA was then

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electrophoresed and visualized on the UV cabinet.

PCR and electrophoresis of transformant candidates of tomato plants

Isolated DNA were amplified in PCR machine with *NPT*II (neomycin phosphotransferase) primer (forward: 5'-GTCATCTCACCTTCCTCCTGCC-3'; reverse: 5' GTCGCTTGGTCGGTCATTTCG-3'), *NPT*II is a kanamycin resistance gene which is the selectable marker in T-DNA construct of *the pKYS-SoSPS*1 plasmid. *NPT*II PCR program with predenaturation 95ºC for 3 minutes, denaturation 95ºC for 30 seconds, annealing 59ºC 30 for seconds, extension 72ºC for 1 minute, final extension 72ºC for 5 minutes (Manders *et al*., 1994). The PCR cycle was performed 35 times with expected target of 550 bp DNA amplified. The result of PCR was electrophoresed then the result was visualized.

RESULTS AND DISCUSSION

NPT*II gene confirmation in *A. tumefaciens

Electrophoresis was performed using agarose. The p*KYS-SoSPS*1 plasmid used as the control. The p*KYS-SoSPS*1 plasmid contains *SoSPS*1 gen to increase the production of plant sucrose (Ningtyas *et al*., 2015) and the *NPT*II gen as kanamycin resistance gene (Ghanem, 2011). The results of the gene confirmation through PCR showed that the tenth single colony samples from *A. tumefaciens* grown on solid LB medium containing the *NPT*II gene which can be seen in Fig. 2. It appears that the tenth single colonies of *A. tumefaciens* amplified positive containing p*KYS-SoSPS*1 plasmid, so it can be ascertained that the tenth single colonies of *A. tumefaciens* used for the transformation in this research positively contain the *SoSPS*1 and *NPT*II genes.

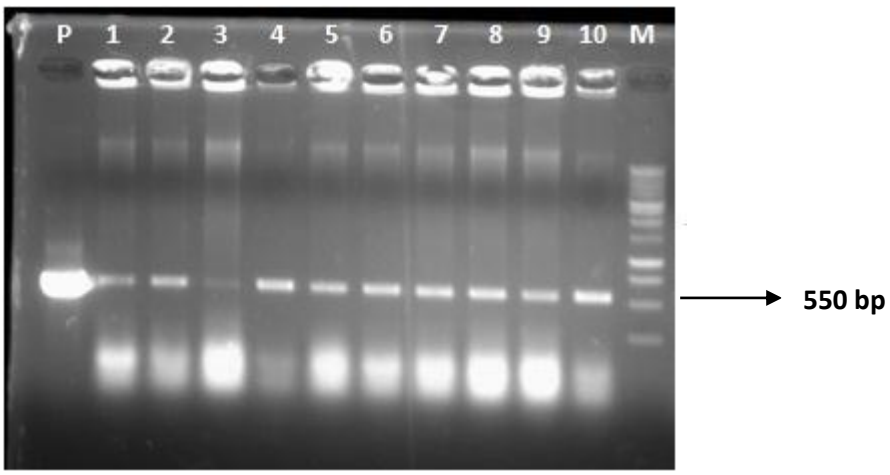

Fig. 2. The gene confirmation in the *A. tumefaciens* using *NPTII* primers.
P = *pKYS-SoSPS*1 plasmid; 1-10=single colonies of *A. tumefaciens*; M=DNA marker of 1 kb DNA ladder.

Percentage of seeds germinated

Seeds that germinated after pricked and soaked were 22% in the 1-day soaking treatment and 18% in 2 days soaking treatment. Germinated seeds after transformed were planted in the soil until 13 days (Fig. 3). The low number of seeds germination caused by the seeds were pricked, this can be concluded that the tomato seeds used in this research has good quality because there were 98% of seeds germinated in control treatment or without pricked.

Fig. 3. Tomato seeds Germination and grow after transformed.
A = Seed germinates after 1-day soaking treatment;
B = Seed germinate after 2-days soaking treatment;
C = Seeds which become plants after the transformation of 1-day soaking treatment;
D = Seeds that become plants after the transformation of 2-days soaking treatment.

Pricking of tomato seeds in the middle with the target endosperm was done to injure and penetrate the seed coat. The wound on the seeds provides access for *A. tumefaciens* into the seeds to infecting the embryo in tomato seeds. The low percentage of the germinated seeds due to the embryo or the endosperm damage. Pricking tomato seeds with a needle may be damaged the embryo so that the seeds are not able to germinate. According to Syukur *et al.* (2012), the embryo in the seed is a tiny plant that will germinate and grow into a plant, so that if the embryo on the seed were damaged it will interfere the seed germination. The growth of the sprouts becomes slow and thin because the prick treatment damages the endosperm. Endosperm in the seed intends as a food or nutrient supplier to maintain the embryo during the process of embryo development

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into sprouts (Sukamto, 2011). Damage to the endosperm in the seed affects the number of nutrients absorbed by the seed for the early development of tomato embryos.

Tomato plant DNA isolation

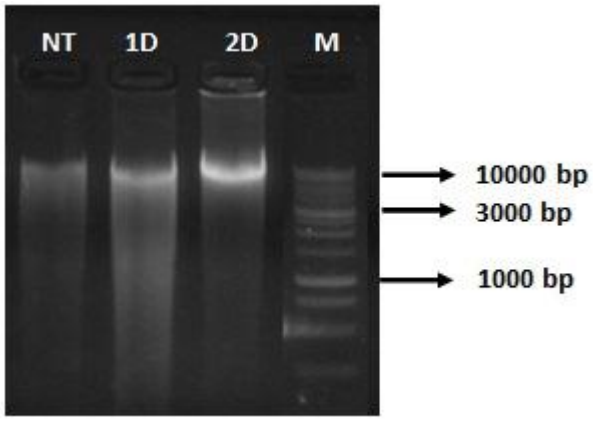

Fig. 4. Visualization of DNA isolation result from tomato leaves samples.
NT = non-transformant sample; 1D = 1 day immersion; 2D = 2 days immersion; and M = DNA marker 1 kb DNA ladder.

Fig. 4 shows that DNA from tomato leaves can be extracted using the modification of Mini-Prep method from Zang and Stewart (2000). The number of samples used in this research only 0.03 grams per sample from the recommended 0.3 grams per sample in the Mini-Prep method. Thus, this method can be used to extract DNA from the leaves of tomato plants with fewer samples needed in the Mini-Prep method by Zang and Stewart (2000).

Molecular analysis of transformant candidates of tomato plants

The results of this research showed that the prick and soak *in planta* transformation with the treatment of 1-day soaking of tomato seeds was able to integrate T-DNA into the tomato plants genome. The integration of the *NPT*II gene with plant genome is also accompanied by the integration of the *SoSPS*1 gene. The use of *NPT*II as a primer because of *NPT*II is a specific gene not owned by tomato plants (Meissner *et al*., 1997).

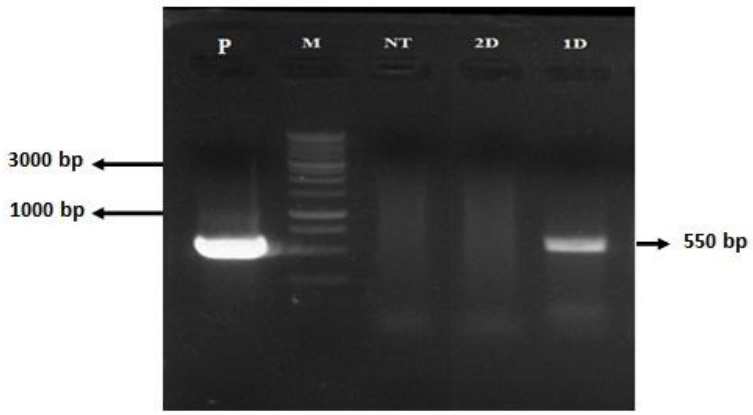

Fig. 5. Electrophoresis visualization of the PCR results from isolated DNA of transformant candidates of tomato plants using primers of the *NPTII* gene. P = *pKYS-SoSPS*1 plasmid, M = DNA marker of 1 kb DNA ladder, NT = non transformed plant, 2D = 2 days soaking treatment, and 1D = 1 day soaking treatment.

In planta transformation has been successfully performed using the floral dip (Ratanasut *et al*., 2017; Bent 2000; Li *et al*., 2010; Rod-in *et al*., 2014; Narusaka *et al*., 2010; Zang *et al*., 2006 Clough and Bent 1998), by soaking the flower of the plant in the liquid *A. tumefaciens* culture; the pin pricking (Razzaq *et al*., 2011; Jaganath *et al*., 2013; Seol *et al*., 2008), by pricking the transformation target with the needle smeared with *A. tumefaciens*; agro-injection (Bratic *et al*., 2007; Orzaez *et al*., 2006; Zia *et al*., 2011), by injecting *A. tumefaciens* culture directly into the plant tissue or organs; vacuum infiltration (Nanasato *et al*., 2013; Bratic *et al*., 2007), by a vacuum to force *A. tumefaciens* get in into plant cells; and polen-tube pathway (Semiarti *et al*., 2012; Shou *et al*., 2002; Song *et al*., 2007; Zang and Luan, 2016), by immersing pollen from male flowers with *A. tumefaciens* culture then breed it with female flowers to produce a transformant progeny. There haven't been any reports of transformation done by pricked and soaked the seeds as was done in this research.

The prick and soak *in planta* transformation method is a combination of pricking pin and floral dip methods. The pricking of the transformant target using the needle adopted from the pin pricking method while the soak of the transformation target with the suspension of *A. tumefaciens* adopted the floral dip method. Pin pricking method is done by smearing sterile needle with *A. tumefaciens* culture then the needle is used to pierce the seed or other plants tissue (Seol *et al*., 2008), while the floral dip method is done by soaking the existing flower on the plant without injuring the flower (Clough and Bent, 1998).

The pin pricking method is considered to be less efficient because the smeared needle has a narrow surface area. This results the number of bacterias carried by the needle are limited, affecting the low chance for transformation. The weakness of the floral dip method lies in the length of time required to obtain the transformant plant. Floral dip method can be done if the plants already have flowers. After transforming the flowers, we still have to wait for the flowers to become fruits to the physiological ripe so that the seeds can be grown to be selected as the candidate transformant plants. The disadvantages of the pin pricking and floral dip methods inspired the prick and soak method. The prick and soak method is performed by prick the transformation target and then soak it with the suspension of *A. tumefaciens*.

Soaking the seeds that have been pricked once with the *A. tumefaciens* suspension done with the aim to provide nutrition, optimal environment to facilitate *A. tumefaciens* to move to the plant cells so that bacterial infections to the plant cells more evenly to all plant cells that submerged have

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the possibility to be infected. According to Dewanto and Suhandono (2016), soaking media is important in transformation because the media supports optimal conditions that can promote the interactions and increase gene transfer rates and weaken plant cells defenses to facilitate bacterial infections. It shows that the use of the soaking media has an effect on transformation.

The time of soaking or inoculation time has an effect on the transformation. According to Dwiyani *et al*. (2016b), inoculation times becomes an important factor in transformation. Soaking for 1 day gives the best result of transformation compared to soaking for 2 days. Allegedly, there is an optimal inoculation or soaking time to obtain a high transformation efficiency, if passing a certain time then the number of transformants obtained will be reduced. Purnamaningsih (2010) in *in vitro* transformation found that the inoculation treatment of tomatoes cotyledon showed the inoculation time for 45 minutes gave the best result from the treatment of 0, 15, 30 and 45 minutes of *A. tumefaciens* inoculation. However, in a study conducted by Shrestha *et al.* (2007) using orchid protocorm *in vitro* with 10, 60, 240 and 480 minutes inoculation treatments showed that the 240-minute inoculation treatment resulted in the highest number of transformants.

The longer interaction between plant cells and *A. tumefaciens* caused the plant to detect the presence of *A. tumefaciens* as a pathogen and begin to increase the resistance system and limit the activity of the transformation by hypersensitive reaction (Coll *et al*., 2011). According to Jones and Dangl (2006), hypersensitive reactions are active reactions when the pathogen interfere with molecular plant cells. The stage begins with the plant recognizing the pathogen that enters and begins to activate the resistance system. Successful pathogens propagate virulent effector. Effector recognized by plant immune systems send signals to nucleotides. Nucleotide response form is resistant to the pathogen by programmed cell death (hypersensitive reaction).

Another thing that supports the success of transformation in this research is the use of tween20 and acetosyringone (AS). Clough and Bent (1998) suggest that the addition of surfactants at low concentrations in inoculation media can increase the number of transformants obtained in *Arabidopsis thaliana* plants using the floral dip method. Tween20 is one of surfactant that serves to reduce the surface tension of the cell so that hydrophobic reactions of plant cells and bacterial cells decreases and facilitates the attachment of *A. tumefaciens* in plant cells (Miller, 2013).

Research conducted by Sheikholeslam and Weeks (1987) in *Arabidopsis thaliana* showed that addition of acetosyringone able to increase the number of transformants up to 63% than without giving acetosyringone which only gets 2-3% transformants. *A. tumefaciens* is a gram-negative bacteria that carries the transfer DNA (T-DNA) in its plasmid and is able to infect through wounds in the plant. The plasmid of *A. tumefaciens* is composed of left border, T-DNA, right border, and virulence region (virgen). AS compounds play a role in activating virgen to release T-DNA from plasmids and deliver T-DNA to plant cells to integrate with plant genomes (Afolabi-Balogun, 2014).

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