Journal of Agricultural Science and Technology A 7 (2017) 393-400
doi: 10.17265/2161-6256/2017.06.004

Pollen Germination and Pollen Tube Elongation of Tomato (*Lycopersicum esculentum* L.) Regulated by Cell Wall Invertase through Sucrose Hydrolysis

Astija

Universitas Tadulako, Kemenristekdikti 94119, Indonesia

Abstract: Sucrose is a sugar required for pollen germination and pollen tube elongation. It is little known on the regulation mechanism. As such, this research was conducted to reveal mechanism pathway of the sugar in regulating pollen germination and pollen tube elongation by cell wall invertase. The pollen grains, respectively originated from wild type and transgenic tomato plants, which had been silenced their inhibitor gene (*INVINH1*) of the cell wall invertase were used in this study. The pollen grains were cultured in media containing glucose, fructose or sucrose. Results of the study showed that percentage of the pollen germination derived from transgenic plants was much higher than that from wild type plants. Moreover, pollen tube elongation was longer in transgenic plants compared with wild type plants. Interestingly, these results were observed in medium containing sucrose only, but not in glucose and fructose. This result suggests that cell wall invertase activity regulates pollen germination and pollen tube elongation through sucrose hydrolysis.

Key words: Sucrose, cell wall invertase, pollen germination, pollen tube elongation, tomato.

1. Introduction

In flowering plants, reproduction process occurs by involving the union of the male cell (pollen) and the female cell [1, 2]. The process is preceded by the occurrence of pollination, a process during which pollen grains come from the sporangium of anther land on the surface of the stigma. The pollen grains are facilitated by pollinator agents, such as the wind, water, animals and humans. Each plant species has one or more special agents. The pollen grains interact with stigma, and undergo germination and elongation. Both processes involve physiological, biochemical and genetic aspects [3-5].

The pollen germination process begins with an event of rehydration or water absorption from papilla surface of stigma through a certain structured site of the pollen grains called “aperture”. At this place, the cell wall structure of the pollen grains is thinner with little exine (outer layer) than in other parts [6]. The rehydration occurs as a result of the hydration experienced by pollen grains after those exit from the sporangium and is present in a hot or dry outdoor environment [7]. The absorption leads to an increase in turgor pressure that can ultimately loosen a plasma membrane and cell wall of the pollen grains [8]. In addition, the absorption stimulates enzymatic activity and metabolism of the pollen grains. The stimulation results in pollen germination [9]. The germination that occurs through the aperture will continue to form the bulge of the plasma membrane and its cytoplasm outward to produce a pollen tube [10]. The pollen tube extends and penetrates the cells of the pistil tissues. This elongation is intended to deliver the generative nuclei to meet the egg nuclei and polar nuclei for the fertilization process that produces the embryo and endosperm, respectively. This process takes place inside the ovule [11].

Several studies have reported that the elongation or growth of pollen tubes to the ovule is regulated and

Corresponding author: Astija, associate professor, research field: plant science.

directed by chemicals or chemotropic substances derived from pistil tissues [12]. These substances, such as sugar, fat and protein, are important regulators in germination and elongation. Sucrose is the final product of photosynthesis which has a crucial role in regulating plant growth and metabolism [13-15]. Sucrose is transported by the vessel element and its companion cell to enter the sink cells in two ways, i.e., the apoplasmic and simplasmic ways. The apoplasmic way is the transportation of substances passing through the cell wall, while the simplasmic way is passing through the plasmodesmata [9]. By the first way, sucrose into the cell wall is transported by a sucrose transporter and hydrolyzed by the cell wall invertase into glucose and fructose, which are then transported into the cytoplasm by a monosaccharide transporter (hexose transporter). Cell wall invertase activity can be inhibited by a protein inhibitor that causes hydrolysis of sucrose into glucose and fructose decreases [16]. The inhibitor protein is located together with the cell wall invertase enzyme in the cell and the inhibitor works posttranslation [17]. On the other hand, the second way (symplasmic transportation) occurs when sucrose enters the cytoplasm through plasmodesmata, a cytoplasmic relationship among cells [18, 19].

Pollen loses its simplasmic transportation. It is likely that the transport of sucrose from the outside into the cell occurs via apoplasmic. This hypothesis is based on a proposed transport model that sucrose enters from extracellular pistil tissues into the cytoplasm through the pollen cell wall [20]. It is further proposed that the sucrose within the cell wall will be hydrolyzed by the invertase enzyme (cell wall invertase) into glucose and fructose. If the hypothesis is proven, the role of the invertase enzyme becomes important in the pollen germination and the pollen tube elongation through the regulation of the breakdown of sucrose and the regulation of the supply of substances in the cells to regulate osmotic pressure, growth, metabolism and sugar signals within the cell.

Recently, the role of invertase enzymes in regulatory processes has been extensively studied in different plant species by altering their genes or inhibitor genes. For example, silencing of *LIN5* gene included into cell wall invertase leads to a reduction in fruit size, increases the rate of abortion of flowers and shows the different structures of fruits and flowers [21]. Conversely, the inhibition of the cell wall invertase inhibitor, *INVINH1*, leads to an increase in cell wall invertase activity, hence a twofold increase in fruit production, delayed leaf aging, increased hexose levels in fruit and seed [16]. In addition, studies conducted on the development of pollen showed that cell wall invertase silencing resulted in abnormal forms of pollen and sterility pollen in tobacco plants [22], and a decrease in pollen germination from tomato plants [21].

This paper aimed to reveal the mechanism of cell wall invertase in regulating pollen germination and pollen tube elongation via sucrose hydrolysis.

2. Materials and Methods

2.1 Genotype Analysis of Transgenic and Wild-Type Plant DNA

The pollens, respectively originated from transgenic and wild-type plants, were used in this study. To verify the genotypes of the two types of plants, genotypic checking was performed using PCR method. Cotyledon of four plants of each type was used to extract genomic DNA to verify the genotypes. Cotyledons with size of 1 cm wide and 2 cm long were excised and placed into a 1.5 mL tube, followed by immediate immersion into liquid nitrogen before storage. Genomic DNA was isolated from the frozen samples by crushing with a tooth pick, and 1 mL of the extraction solution was added before tubes were centrifuged at 10,000 ×g for 10 min. Extraction solution consisted of 0.35 M glucose, 0.1 M Tris-HCl, 50 mM Na_2EDTA, 2% PVP, 1% β-Me and ddH_2O. The supernatant was discarded and the pellet was added to a 600 μL lysis solution. The lysis solution

consisted of: 1.4 M NaCl, 0.1 M Tris-HCl, 20 mM Na_2EDTA, 2% CTAB, 2% PVP, 1% β-Me and ddH_2O. The lysis process was conducted using incubating at 65 °C for 30 min turning over every 10 min. To separate DNA from the dissolved protein or other materials, the solution was then added to a 600 µL solution mixture consisting of chloroform and isoamyl alcohol with a proportion of 24:1. The solution was again centrifuged and dissolved with chloroform in isoamyl alcohol (24:1). The solution was shaken 30 times and centrifuged at 11,000 ×g for 10 min. The DNA-containing resultant solution was transferred (500 µL) into a new tube to which 500 µL of chloroform in isoamyl alcohol (24:1) was added. After mixing and centrifuging at 11,000 ×g for 10 min, the DNA, upper layer, was then transferred (400 µL) into a new tube. To precipitate the DNA, the solution was then added to 40 µL sodium acetate and 240 µL isopropanol. The solution was kept at -20 °C for 15 min or continued immediately to be centrifuged at 11,000 ×g for 10 min. The supernatant solution obtained was then discarded and added 1 mL ethanol (70%). Thereafter, the solution was mixed and centrifuged at 12,000 ×g for 3 min. The precipitated DNA was washed by using ethanol (70%) repeated three times and dried for 5 min. The purified DNA was then added 50 µL ddH_2O for smaller DNA or 100 µL ddH_2O for bigger DNA. The DNA was kept at -20 °C or used immediately to check its quality

PCR was conducted using INH primers designed using PRIMER-BLAST from NCBI. The primary pair obtained from the program was forward primer: TGTTGGTAGAGCCATTGTAAGA and primary reward: GGGGATACACACATAACATTTGAGG, with each TM 57.1 for forward primer and 59.7 for primary reward, and the size of the expected product was 200 bp. The primer will detect the *INVINH1* of genomic DNA of wild type and control plants, which encodes a protein inhibitor of the cell wall invertase enzyme. The control plant was used for positive control in that the plants that had been known the gene. In addition, negative control were used using the primer added without genomic DNA (or water) in the mixture.

The transgenic plants were a silenced-inhibitor gene (*INVINH1*) of cell wall invertase enzyme through RNA interference (RNAi) technique [16].

2.2 In Vitro Germination and Elongation Assay

By the genotypes analysis, each of the four plants used as samples in this study has been ascertained origin from transgenic or wild-type plants. Furthermore, these plants were then kept growing in the greenhouse. Plants began to enter the reproductive phase, when they have reached two and a half months with the appearance of the inflorescence. Once entering the anthesis stages, pollen grains from a sporangium were removed by vibrating the flower stalk with a vibration device (Vibrator).

The pollen grains of each flower were collected into a tube sized 1.5 mL and of which was added 1 mL of distilled water. After stirring for 15 s, 20 µL aliquotes of these pollen suspensions were cultured in 1 mL media with a mixture of 20 mM MES, 60 mM PEG 4000, 2.964 mM $Ca(NO_3)_2{\cdot}4H_2O$, 0.811 mM $MgSO_4{\cdot}7H_2O$, 0.989 mM KNO_3, 1.617 mM H_3BO_3 with the addition of one of 58.42 mM sucrose, glucose or fructose.

The germinating pollen was indicated by a cytoplasmic outcrop that comes out of the aperture of the pollen wall by reaching less than or equal to the diameter of the pollen. The diameter of the pollen from tomato plants has an average size of 20 µm [23] . Pollen germination and pollen tube elongation of four individual plants of each treatment were observed for 3 h at 25 °C. Using a microscope, the percentage of the pollen germination was calculated by comparing pollen germinated with the total of the polen grains (pollen germinated + pollen un-germinated), while the length of the pollen tube was calculated by using the ImageJ program.

Then, changing the different sugar concentrations

with 29 mM sucrose, 58 mM glucose and 58 mM fructose was conducted to examine whether the effect of cell wall inverase activity on pollen germination and pollen tube elongation was dependent on sucrose only. The used methods were similar to pollen germination and pollen tube elongation methods as described above.

The significant differences of the pollen germination percentages and length of the pollen tubes were analyzed by ANOVA one way using JMP version 11 program.

3. Results and Discussion

3.1 Genotype Analysis

From the PCR assay, it was found that four wild type plants had the expected genotype of the wild type by showing the DNA bands of the *INVINH1* gene. While the four plants of the transgenic plants did not get the DNA band for the *INVINH1* gene (Fig. 1).

3.2 Sucrose Promotes Pollen Germination and Pollen Tube Elongation

The growth of the pollen tubes in a liquid medium added fructose, glucose and sucrose was shown in Fig. 2. Pollen grains cultured in media containing fructose (Fig. 2a) showed that there was still a lot of un-germinated pollen, compared to those in media containing glucose and sucrose. Additionally, the pollen tube in the fructose media (Fig. 2a) elongated shorter than those in the glucose (Fig. 2b) and sucrose media (Fig. 2c). Even the pollen tubes in media containing sucrose were the longest. These results indicated that sucrose promotes pollen germination and pollen tube elongation.

To determine the percentage of the pollen germination, number of pollen germinated to total of the pollen was calculated. Results showed a significant difference between the pollen germination of transgenic and wild type plants from medium containing sucrose. Percentage of the pollen germination coming from transgenic plants was higher than that from wild plants. Compared to medium containing glucose and fructose, the pollen germination in the sucrose medium was the highest (Fig. 3).

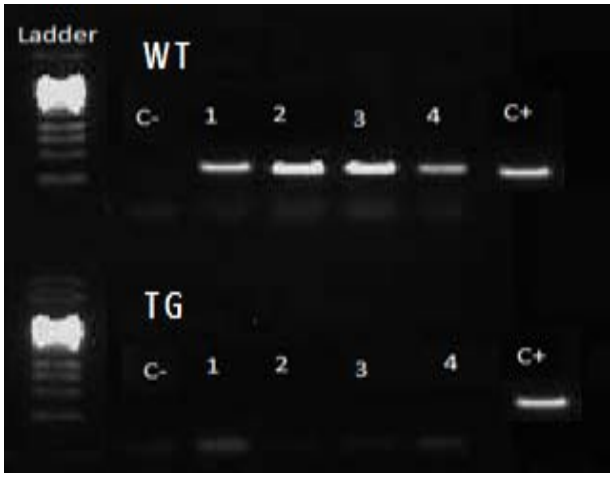


Fig. 1 Genotype analysis of transgenic (TG) and wild-type plant (WT) DNA.

INVINH1 gene was detected in all four wild type plants and the positive control plants (C+) but not detected in all four transgenic plants and the negative control (C-).

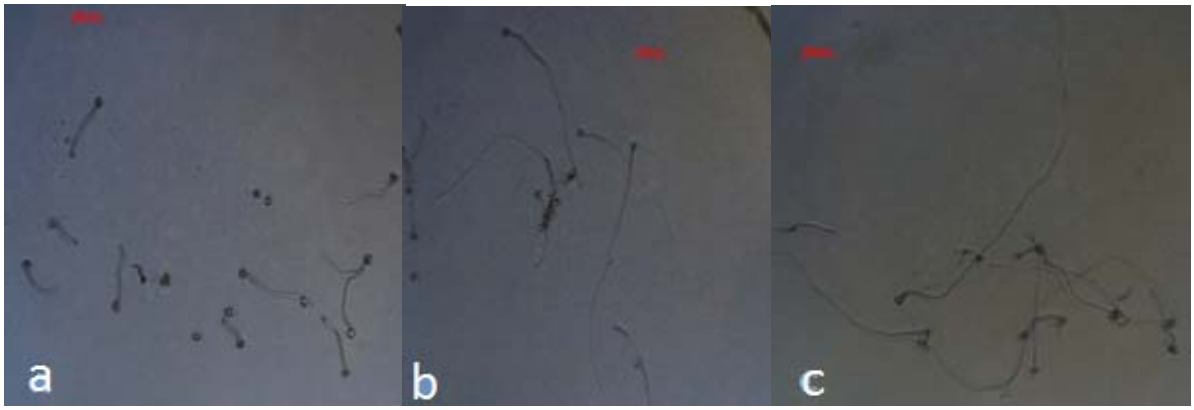


Fig. 2 The growth of the pollen tubes in a liquid medium added fructose (a), glucose (b) and sucrose (c).

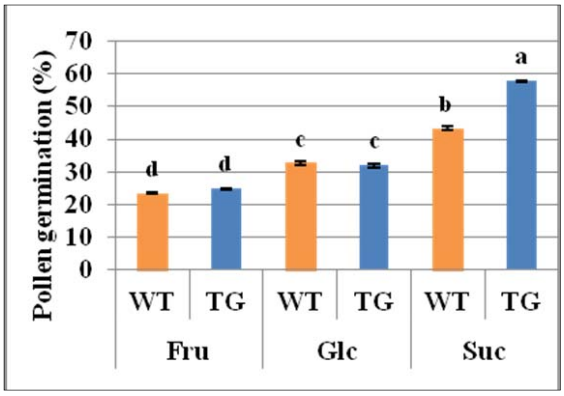


Fig. 3 Pollen germinations of wild-type (WT) and transgenic (TG) tomato plants incubated in medium containing fructose (Fru), glucose (Glc) or sucrose (Suc).
Data represent mean ± SE; different letters indicate significant differences in ANOVA with $\alpha = 0.05$.

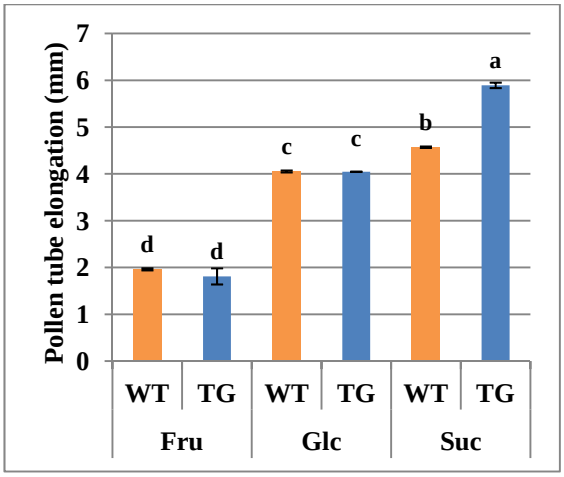


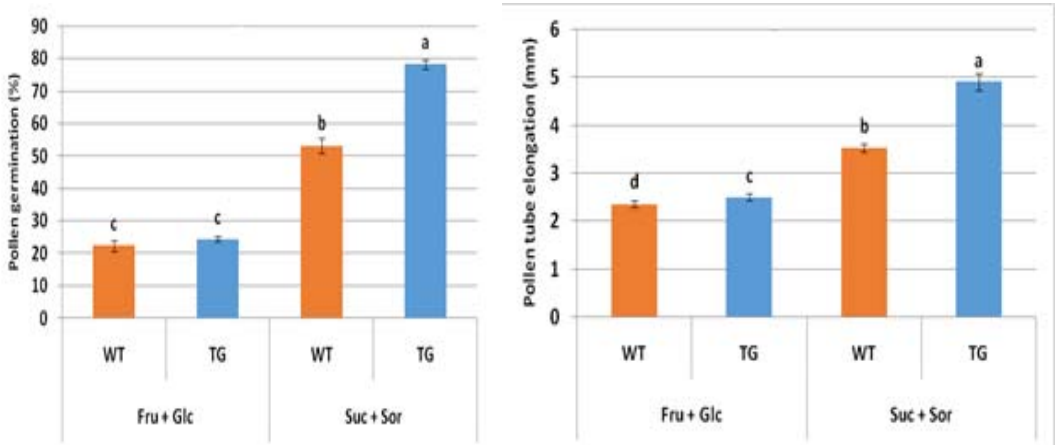
Fig. 4 Pollen tube elongation of wild-type (WT) and transgenic (TG) tomato plants incubated in media containing fructose (Fru), glucose (Glc) or sucrose (Suc).
Data represent mean ± SE; different letters indicate significant differences in ANOVA with $\alpha = 0.05$.

Similar to pollen germination, the pollen tube elongation of the transgenic plant was longer than those from the wild-type plants. This phenomenon also occurred in medium containing sucrose only, but it did not occur in glucose and fructose media (Fig. 4).

From those experiments mentioned above, sucrose plays an important role in the regulation of pollen germination and the pollen tube elongation. Sucrose may play a role in these processes to provide a carbon source for growth and development, in which the sucrose consists of glucose and fructose. These experiments also prove that cell wall invertase affected the pollen germination and pollen tube elongation, in which the pollen grains originated from the transgenic plants germinated and elongated longer than those from wild-type plants. These occur on sucrose medium only. As such, it suggests that cell wall invertase regulates the pollen germination and pollen tube elongation through sucrose hydrolysis. This result support the proposed model that sucrose transported into pollen tube is hydrolyzed by invertase enzyme into glucose and fructose in cell wall [20]. This result is also consistent with the previous reports that sucrose stimulated pollen germination [24-28]. Interestingly, this result suggests that sucrose enhanced these processes through sucrose hydrolysis to glucose and fructose by the activity of invertase enzyme. According to several previous studies, glucose and fructose from sucrose hydrolysis by the cell wall invertase play a very important role in cell metabolism, signal molecules and regulators in the regulation of gene expression [14, 29-36]. Furthermore, the resultant sugars of sucrose hydrolysis must be transported to the cytoplasm by a monosaccharide transporter [37-40].

Additionally, it was demonstrated that pollen germination and pollen tube elongation were regulated by sucrose hydrolyzed by cell wall invertase, but not by differences in the added sugar concentrations. This reason is based on a concept that sucrose consists of glucose and fructose, which makes it have two times larger of osmotic pressure. To verify the hypothesis, a similar study was conducted by changing the different sugar concentrations with 29 mM sucrose, 58 mM glucose and 58 mM fructose. Apparently, the results obtained were similar to those of previous experiments, namely, the pollen germination and pollen tubes elongation from transgenic plants occurred greater than those from wild-type plants in a medium containing sucrose (Fig. 5).

The results of the experiment ascertain that the



(a) Percentage of pollen germination (b) Length of pollen tube

Fig. 5 Percentage of pollen germination (a) and length of pollen tube (b) in media added with a mixture of fructose and glucose and a mixture of sucrose and sorbitol.

Data represent mean ± SE; different letters indicate significant differences in ANOVA with $\alpha = 0.05$.

role of sucrose could not be replaced by a mixture of glucose and fructose. As shown in Fig. 5 that the pollen germination and pollen tube elongation remain higher in sucrose (+ sorbitol) than in the glucose and fructose media. It means that the sucrose is highly required in the process of the germination and elongation of the pollen. Note that sorbitol added with sucrose acts as an osmotic balance only, because it could not be metabolized. The results of the study support several views that sugars generated from photosynthesis and transported to sink cells including pollen and pollen tube were in a sucrose form [13-15, 20, 25]. It is also proposed that apoplasmic-transported sucrose will be decomposed by invertase into glucose and fructose, and the monosaccharide may be transported into the cytoplasm by monosaccharide transporter [20].

4. Conclusions

From this study, it can be concluded that sucrose enhances germination of pollen and elongation of the pollen tube through a sucrose hydrolysis by cell wall invertase mechanism. It is recommended to investigate a regulation of pollen germination and pollen tube elongation in pistilous tissues (*in vivo*) by the cell wall invertase.

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