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Heat Temperature Suppresses Cell Wall Invertase Activity within Sucrose Hydrolysis on Pollen Tube of Tomato

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ABSTRACT: *Pollen, male gametophyte, is required for successful pollination to produce fruit- and seed-set. Pollination is highly vulnerable to abiotic stresses such as heat that often lead to fruit- and seed-abortion and hence yield loss. During that process, pollen germination and tube growth within pistilous tissue to deliver the sperm cells to the ovules. Their growths are highly sensitive to heat temperature and are regulated by sugar. In order to study these processes, we investigated the cell wall invertase (CWIN) regulation of in vitro-grown tomato pollen tubes, under normal and heat stress condition using RNAi-mediated transgenic plants that CWIN inhibitor was silenced and CWIN was silenced. Results showed that the elevating CWIN activity increased the pollen germination rate and pollen tube elongation. By contrast, the repression of CWIN decreased the pollen germination rate and pollen tube elongation. The regulation of CWIN was stimulated by sucrose but it was reduced by heat stress.*

Key words: *Heat stress, Cell wall invertase, Pollen tube, sucrose hydrolysis.*

I. INTRODUCTION

Pollen is male gametophyte which is required for sexual reproductive in flowering plants. It is produced by anther, male reproductive organ. Mature pollen is released and reaches on stigma of pistil. The pollen germinates to form pollen tube, a tip-growing cell. The pollen tube grows through stylous tissue into ovule to deliver two sperms. In the ovule, the sperms within pollen tube are discharged into embryo sac through an interaction between pollen tube and embryo sac. One of these two sperm cells fertilizes egg to form embryo and another sperm fuses with central cell to form endosperm (Braun, 2022; Du et al., 2020; Julius et al., 2017). During the development, pollen is very sensitive to high temperature. Some studies have reported that elevated temperature on pollen development causes male sterility (Hirsche et al., 2009; Jain et al., 2007, 2010) and reduction of pollen viability and pollen germination (Zanor et al., 2009). The reduction is due to disruption of sugar metabolism (Pressman et al., 2002). In sugar metabolism process, sugar hydrolysing enzymes are apparently critical role.

Two important enzymes involved in those processes are invertase and sucrose synthase. The former, enzyme hydrolyses irreversibly sucrose into glucose and fructose (Cordoba et al., 2015; Hasanuzzaman et al., 2013; Hirose et al., 2014; Wahid et al., 2007). The later, enzyme cleaves reversibly sucrose into UDP glucose and fructose (Jain et al., 2008; Kandel-Kfir et al., 2006; Liu et al., 2013; Park et al., 2010; Ruan, 2014). There are three type of invertase, based on subcellular and pH optima. Those are cell wall invertase (CWIN), cytoplasmic invertase (CIN), and vacuolar invertase (VIN) (Ruan, 2014).

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Sucrose is synthesised photosynthetically by active source tissues. It is then exported and transported via phloem into pollen. The sucrose is the major sugar not only as initial substrates providing carbon sources and energy metabolism for pollen development but also as signal molecule in regulating of gene expression (Briefing, 2007; Krichevsky et al., 2007). Several studies have demonstrated that sucrose added into a medium in culture system raised pollen tube growth rate. As a consequence, the sucrose is a crucial sugar for regulation of pollen tube growth.

Invertase is believed to play a key role in the regulation of pollen development. Given that pollen is a single cell and symplasmically isolated so that sucrose hydrolysis takes place apoplasmically. The hydrolysis is conducted by CWIN, enzyme which is bound ionically in cell wall (De Storme and Geelen, 2014; Swanson and Gilroy, 2010). Several studies have reported that suppression of the CWIN results in aberrant pollen morphology, the male sterility and reduction of pollen viability and germination (Goetz et al., 2001; Zanor et al., 2009). Expression and activity of CWIN is regulated by a number of stimuli such as heat stress and sugars (Roitsch et al., 2003). Down-regulation in CWIN activity was induced by heat stress (Pressman et al., 2002).

Effects of temperature on the invertase and sugars on pollen development are well studied. However, it is unknown how the underlying mechanism is on elongating pollen. This study investigated the phenotypes of pollen development with the emphasis of pollen germination and pollen tube elongation. This investigation used transgenic plants which had been successfully transformed with an RNA interference (RNAi) silencing against *INVINH1* (Jin et al., 2009) and *CWIN* (Zanor et al., 2009). In vitro study was conducted to examine the effects of a certain type of sugars i.e. sucrose, glucose, and fructose and heat temperature on pollen germination and pollen tube elongation.

Our results showed that the elevating CWIN activity increased the pollen germination rate and pollen tube elongation. By contrast, the repression of CWIN decreased the pollen germination rate and pollen tube elongation. The activity of CWIN was stimulated by sucrose but it was reduced by heat stress.

II. MATERIAL AND METHODS

Plant growth

Plants of tomato (*Solanum lycopersicum*) which were used in this study included transgenic plants; INH-RNAi and CWIN-RNAi, with their wild type; WT-1 and WT-2, respectively. Several seeds of each plant type were grown in a pot sized 265 mm. A mixture of used soil consisted of coarsie sand, perlite, and coir peat in ratio of 2:1:1 respectively. The growing seedlings of the plants were selected having uniform growing. The procedure was detailed in section 2.2.2. Once the plants had grown two leaves at an age of 4 weeks, they were selected and transplanted into other pots. The plants were fertilised twice, once aged 6 and the second time at 12 weeks of age. The used fertiliser consisted of osmocote standard and osmocote high potassium in ratio of 1:1. A weekly liquid fertiliser regime of jurox wuxal liquid foliar feed was also used. The plants were grown under normal controlled condition (day/night temperature of 25°C /18°C) with 12-h light photoperiod from 06.00 am to 06.00 pm in the glasshouse. Plants were watered once a day every evening using automated drip irrigation.

Pollen germination media

The pollen germination medium according to Astija (2017) with little modification that was chemical substances consisting of 20 mM MES, 60 mM PEG 4000, 2.964 mM $Ca(NO_3)_2$. $4H_2O$, 0.811 mM $MgSO_4$. $7H_2O$, 0.989 mM KNO_3, 1.617 mM H_3BO_3. Those substances were placed in a volumetric flask and were added distilled water until reached 950 ml in volume. The solution was shaken and made up to a volume of 1000 ml then transferred to a beaker and shaken for a few seconds. The pH value of the solution was raised to pH 6 by the drop wise addition of a 1M NaOH solution with continuous stirring (magnetic stirrer). This stock solution was used to produce pollen germination and a pollen tube growth medium containing either 58.428 mM sucrose, 58.428 mM glucose or 58.428 mM fructose.

Pollen germination and pollen tube elongation was observed by placing 1 µl of the pollen medium on a microscope slide. The images were taken of the pollen tube until precisely 100 pollen tubes on a Zeiss Axiocam digital camera using Axiovision V4.8 software. The observation of pollen germination was conducted by placing

a small petri dish sized 35 mm x 10 mm under a microscope. This observation was repeated three times with the petri dish being situated in a different location of petri dish for each observation under the microscope for each of the four biological replicates. Percentages of germinated pollen grain were determined by calculating in proportion between a number of the protrusion of tube which was at least equal or greater than the diameter of pollen and total of the observed pollen. The images of the pollen tube elongation, however, were measured with the aid of a computer ImageJ1.46r analysis program.

Data analysis

The data obtained from the pollen germination and pollen tube elongation were analysed using a one-way ANOVA. Differences in all pairs of the temperatures, sugars, and the types of plants were used with the Tukey HSD analysis with (P = 0.01), JMP software procedure.

III. RESULTS

Wild type and INH-RNAi transgenic results of pollen grain germination were shown in Figure 4. Germination rates of wild type and transgenic plant (INH-RNAi) pollen grains at 25 °C were significantly greater than those at 35 °C in all sugar media. Comparison between wild type and INH-RNAi germination rates of their pollen grains revealed that there were no significant difference in pollen grains cultured on fructose and glucose at 25 °C or 35 °C. For sucrose, however, rates of germination for transgenic plants (INH-RNAi) pollen grains were higher than those of wild type pollen grains at 25 °C and 35 °C (Figure 1).

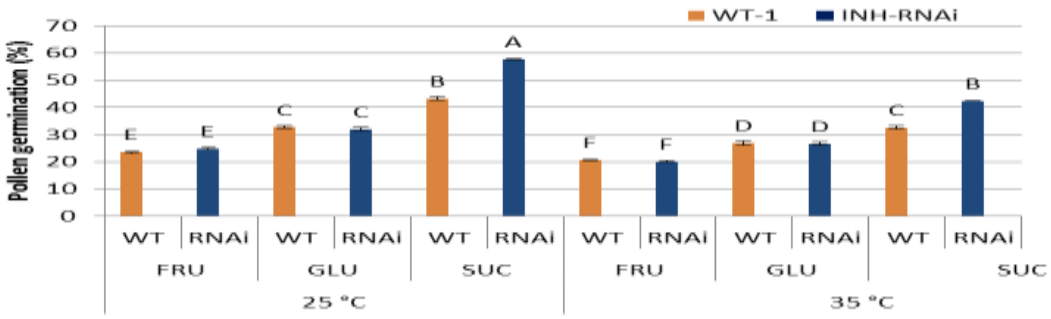

Figure 1. Pollen germination rate of wild type and INH-RNAi transgenic plants cultured in fructose (58.428 mM), glucose (58.428 mM), and sucrose (58.428 mM) media at temperature 25 °C and 35 °C with a duration of four hours. Fru (frucose), Glu (glucose), Suc (sucrose), WT (wild type plants), RNAi (INH-RNAi transgenic plants). Error bars represented ± SE, different letters indicated significant differences in ANOVA analysis with α = 0.01.

Figure 2 illustrated that pollen tube elongation of wild type and INH-RNAi transgenic plants, which were cultured at 25 °C, were significantly longer than those at 35 °C. The pollen tube elongation of INH-RNAi, compared with wild type, was also significantly longer in sucrose medium. However, the pollen tube elongation was not significant differences in fructose and glucose media both at 25 °C and 35 °C.

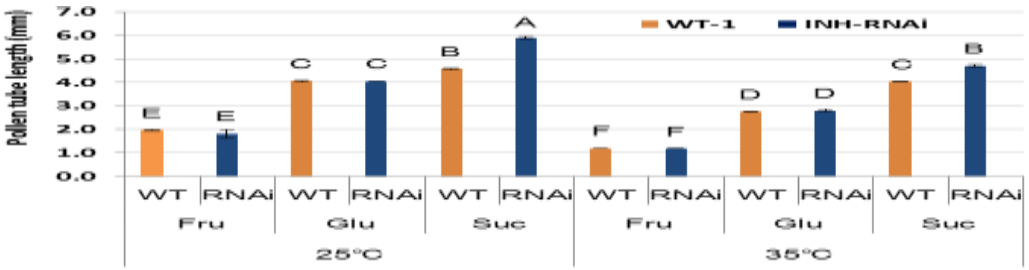

Figure 2. Pollen tube elongation of wild type and INH-RNAi transgenic plants cultured in fructose (58.428 mM), glucose (58.428 mM), and sucrose (58.428 mM) media at temperature 25 °C and 35 °C during three hours. Fru (frucose), Glu (glucose), Suc (sucrose), WT (wild type plants), RNAi (INH-RNAi transgenic plants). Error bars represented ± SE, n = 100 pollen tubes. Different letters indicated significant differences in ANOVA analysis with α = 0.01.

Wild type and CWIN-RNAi transgenic results of pollen grain germination were shown in Figure 3. Germination rates of the wild type and the transgenic plant under normal condition (25 °C) were significantly greater than those under heat stress (35 °C) in all sugar media. There was no significant difference in pollen germination cultured on fructose and glucose at 25 °C or 35 °C both wild type and transgenic plants. However, rates of germination cultured on sucrose for wild type plants pollen grains were higher than those of CWIN-RNAi pollen grains at 25 °C and 35 °C.

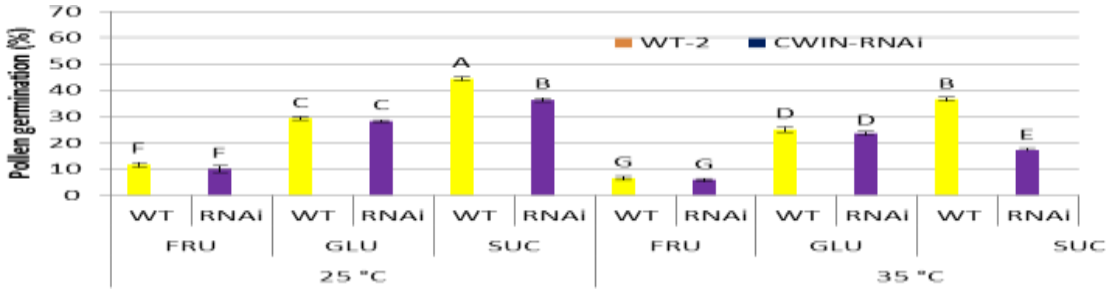

Figure 3. Pollen germination rate in wild type and CWIN-RNAi plants cultured in fructose (58.428 mM), glucose (58.428 mM), and sucrose (58.428 mM) media at temperature 25 °C and 35 °C with a duration of four hours. Fru (fructose), Glu (glucose), Suc (sucrose), WT (wild type plants), RNAi (CWIN-RNAi transgenic plants). Error bars represented ± SE. Different letters indicated significant differences in ANOVA analysis with α = 0.01.

The wild type and transgenic plants (CWIN-RNAi) results of pollen tube elongation which were cultured at 25 °C were significantly longer than those at 35 °C in all sugar media (Figure 4). Comparison of the pollen elongations between the wild type and the transgenic plants (CWIN-RNAi) did not have significant differences in fructose and glucose at 25 °C and 35 °C. In contrast, the pollen elongations in sucrose medium had significant differences in the wild type and the CWIN-RNAi transgenic plants both temperatures at 25 °C and 35 °C. The tube elongation of wild type was longer than that of CWIN-RNAi (Figure 4).

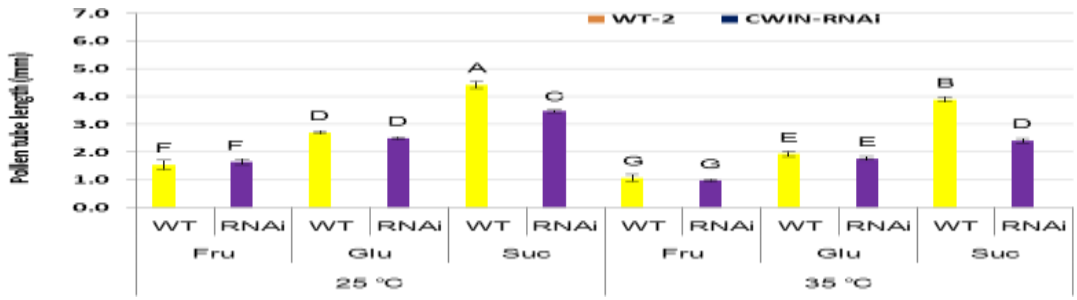

Figure 4. Pollen tube elongation cultured in fructose (58.428 mM), glucose (58.428 mM), and sucrose (58.428 mM) media at temperature 25 °C and 35 °C during three hours from wild type and CWIN-RNAi transgenic plants. Fru (fructose), Glu (glucose), Suc (sucrose), WT (wild type plants), RNAi (CWIN-RNAi transgenic plants). Error bars represented ± SE, n = 100 pollen tubes, different letters indicated significant differences in ANOVA analysis with α = 0.01.

IV. DISCUSSION

Pollen germination rate and pollen tube elongation were significantly greater in the transgenic plants whose CWIN-up regulated than in transgenic plants whose CWIN-down regulated or their Wild type. These

results suggested that the CWIN played crucial role in regulation not only in pollen germination but also in pollen tube elongation. Silencing CWIN using CWIN-RNAi approach reduced the pollen germination and pollen tube elongation. These results partially supported to the previous finding, which demonstrated that suppression of CWIN reduced pollen germination in tomato (Zanor et al., 2009), in tobacco (Goetz et al., 2001). Additional result was that the suppression of CWIN reduced pollen tube elongation. Moreover, elevating CWIN by silencing its inhibitor using transgenic RNA interference approach increased pollen germination and pollen tube elongation.

As known that CWIN played a crucial role in hydrolysing apoplasmically sucrose into glucose and fructose. The monohexoses were then transported via monohexoses transporter into cytoplasm (Fotopoulos, 2005; Le Roy et al., 2013; Zhang et al., 2006). In cytoplasm, the monohexoses was used for respiration, osmotic regulation, and biosynthesis. Alternatively, sucrose was transported by sucrose transporter (SUT) into cytoplasm (Hackel et al., 2006; Sivitz et al., 2008). Then, the sucrose was either hydrolysed into glucose and fructose by cytoplasmic invertase (CIN) or transported into vacuole to store or to hydrolyse by vacuolar invertase (VIN) into glucose and fructose. Those results revealed that the CWIN activity was regulated by sucrose molecule. Pollen germination and pollen tube elongation cultured in sucrose medium were greater than those were in glucose or fructose. These results were parallel with previous several studies demonstrated that sucrose molecule promoted pollen germination and pollen tube elongation in some species in vitro. Indeed, CWIN regulated pollen germination and pollen tube elongation depends on sucrose but not on glucose and fructose (Astija, 2017). These results indicated that sucrose molecule was highly required for regulating the pollen germination and pollen tube elongation. The sucrose appeared that it acted as signal molecule in regulation of CWIN activity for the pollen germination and pollen tube elongation. However, its regulatory mechanism was not still known yet.

Compared to fructose, glucose stimulated slightly pollen germination and pollen tube elongation as shown Figure 1, 2, 3, 4. The stimulation of glucose to pollen germination and elongation in culture system had been demonstrated some workers (Hackel et al., 2006; Schneidereit et al., 2003). In contrast, the inhibition of fructose to pollen germination and pollen tube elongation had been studied in pear (Okusaka and Hiratsuka, 2009). Interestingly, the results demonstrated that pollen germination cultured in glucose was not significant differences in INH-RNAi and CWIN-RNAi while pollen tube elongation in both plants was significant differences. The pollen tube length of INH-RNAi plants reached 4 mm. That was longer than pollen tube length of CWIN-RNAi plants sized 2.5 mm as shown Figure 2 and Figure 4. That meant that the glucose triggers CWIN activity in regulating pollen tube elongation but not in pollen germination. To date, glucose molecule can act a signal molecule regulating the expression of a large number of genes related to the metabolism of carbohydrate, stress response, cell growth, signal transduction, transcription factors, and secondary metabolism (Price, 2003). In plants, the glucose derived from sucrose which was hydrolysed invertase. The result assumed that the exogenous glucose could act as a signal molecule regulating gene expression related to sugar hydrolysing enzymes such as invertase.

The results also demonstrated that CWIN activity in regulation the pollen germination and pollen elongation was affected by temperature. Heat stress reduced the activity of CWIN. The results was agreement with previous workers reported that heat stress reduced the activity and expression of CWIN (Pressman et al., 2012). The pollen germination and pollen tube elongation were reduced under heat stress, in comparison to those under normal condition. The results had also been studied by several workers (Firon et al., 2006; Pressman et al., 2002). Heat stress results in decrease respiration activity (Karapanos et al., 2010). However, this finding suggested that the effect of heat stress on pollen germination and pollen elongation correlated with CWIN activity. The heat stress reduced CWIN activity resulting in reducing pollen germination and pollen tube elongation. Interestingly, compared to wild type plants, INH-RNAi transgenic plants were more tolerant in which the pollen germination and pollen elongation were greater than those of the wild type plants. In contrast, the pollen germination and pollen tube elongation were impaired when the CWIN was silenced under 35 ^{o}C. This means that the CWIN plays a role in response to unfavourable environment such as heat stress.

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