cDNA PROBE DEVELOPMENT FOR SCREENING A DROUGHT TOLERANCE CONTRIBUTING TRAIT IN MAIZE POPULATION

PISCHANAN LOWANTHA

MASTER OF SCIENCE IN AGRONOMY

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PISCHANAN LOWANTHA

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
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ชื่อเรื่อง การพัฒนา cDNA probe เพื่อตรวจสอบลักษณะส่งเสริมความทนแล้งในประชากรข้าวโพดเลี้ยงสัตว์
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บทคัดย่อ

ข้าวโพดเลี้ยงสัตว์ (*Zea mays* L.) เป็นพืชที่มีความสำคัญทางเศรษฐกิจในทุกประเทศ ถูกนำมาใช้ในอุตสาหกรรมอาหารสัตว์เป็นจำนวนมาก แต่ปัจจุบันผลผลิตไม่เพียงพอต่อความต้องการใช้ เนื่องจากได้รับผลกระทบจากสภาวะแล้ง หนึ่งในวิธีการลดการสูญเสียผลผลิตคือการพัฒนาพันธุ์ข้าวโพดที่ทนแล้งขึ้นอาจช่วยเหลือเกษตรกรได้ โดย Trehalose-6-phosphate synthase (TPS) เป็นเอนไซม์ที่สำคัญในกระบวนการสังเคราะห์น้ำตาล Trehalose ซึ่งพบว่าจะสังเคราะห์เพิ่มขึ้นและส่งเสริมความทนทานให้แก่พืชเมื่ออยู่ในสภาพเครียด ดังนั้น งานวิจัยนี้มีวัตถุประสงค์เพื่อคัดกรองระดับการเปลี่ยนแปลงการแสดงออกของยีน *TPS* ในประชากรข้าวโพดเลี้ยงสัตว์ภายใต้สภาพขาดน้ำด้วยการใช้ cDNA probe ผ่านเทคนิค dot-blot hybridization โดยทดลองปลูก 34 S_2 maize families ภายใต้สภาวะเครียดน้ำและเก็บตัวอย่างใบที่จำนวนวันหลังปลูก (DAP) ที่แตกต่างกันเป็นจำนวน 6 ครั้ง เพื่อใช้ในการวิเคราะห์ dot blot assay จากการศึกษาพบว่า ระดับการแสดงออกของยีน *TPS* สูงสุดที่ 4 วัน (Relative intensity at 64 DAP; RI_{64}) หลังจากได้รับความเครียดจากการขาดน้ำ นอกจากนี้ยังพบว่า เมื่อข้าวโพดได้รับความเครียดตั้งแต่ 6 วันขึ้นไป จะสามารถทำให้คัดแยกประชากรข้าวโพดเลี้ยงสัตว์ที่แสดงความทนทานและอ่อนแอออกจากกันได้ และในการศึกษานี้ยังพบอีกว่า ข้าวโพดเลี้ยงสัตว์ที่มีการแสดงออกของยีน *TPS* สูงมักจะทนต่อความเครียดจากการขาดน้ำได้น้อยกว่า ซึ่งเป็นที่น่าสังเกตว่า การแสดงออกของยีน *TPS* ในข้าวโพดเลี้ยงสัตว์ที่โตเต็มที่ภายใต้สภาวะเครียดนั้น จะแตกต่างกับการรายงานก่อนหน้าในระยะต้นกล้าของพืชชนิดอื่นและพบว่า 4 ใน 34 S_2 maize families อาจมีศักยภาพในการทนทานต่อสภาพเครียดจากการขาดน้ำ ซึ่งอาจจะถูกนำไปใช้ในโครงการปรับปรุงพันธุ์ต่อไป

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ABSTRACT

Field maize is an important economic crop and it has been used in the animal feed industry. Maize yields have been inadequate for the demand due to drought events. One way to alleviate yield losses is to develop drought tolerant maize varieties. Trehalose-6-phosphate synthase (TPS) is an important enzyme involved in trehalose biosynthesis which has been found to increase plant tolerance to abiotic stresses. The aim of this research was to screen the levels of *TPS* gene expression in maize breeding materials under water stress via dot-blot hybridization using cDNA probe. To do so, 34 S_2 maize families were grown and subjected to water stress condition. Leave samples were collected at 6 different days after planting (DAP) for a dot blot assay. The results showed that the level of *TPS* gene expression was highest at 4 days after stress. However, dot blotting at 6 days after stress was effective to differentiate maize families. Our study showed that maize with high *TPS* gene expression tended to be less tolerant to water stress. It is noteworthy that the study of *TPS* gene expression in mature maize under stress in this study showed results that contrasted with previous reports on seedlings in many plant species. Furthermore, we found that 4 out of 34 S_2 maize families may have potential for further use in our breeding program.

Keywords : cDNA probe, stay-green phenotype, image processing, relative signal intensity, S2 maize families

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Pischanan Lowantha

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CHAPTER 1

INTRODUCTION

Field maize (*Zea mays* L.) is an important economic crop in Thailand. It has been used as a raw material in the animal feed industry. The demand for field maize has been increasing not only in Thailand but foreign countries. Over the past several years, overall yield decreased from 4.82 million tons in the year 2017/2018 to 4.78 million tons in the year 2021/2022 which led yield per year decreased by 0.7% because of drought and fall armyworm infestation (Office of Agricultural Economics, 2021b). Drought stress is one of the main environmental problems that affect plant growth and yield. Irregular rainfalls have been found through growing seasons resulting in insufficient water for cultivation (Thaitad, 2015) and affected maize growth development and yields.

Maize varieties with drought tolerance could be helpful to mitigate yield losses caused by drought stress. For the plant breeding perspective, an important step of successful breeding work is the selection, especially selection for drought tolerance contributing traits in this case. Conventional breeding is primarily based on phenotypic selection. Integration of molecular techniques with phenotypic selection could shorten time and increase accuracy of selection. Some molecular techniques, such as hybridization assay, could be used to tested many samples simultaneously. This is even suitable for a purpose of sample screening, which need to be simple and rapid.

Hybridization methods have been accepted as a standard technique to detect particular sequences of either DNA or RNA, including western blot hybridization for detecting protein. Dot blot assay is one of the hybridization techniques which detect both DNA and RNA samples. Complementary single strand DNA (cDNA) could hybridize with the single strand mRNA of interest under the optimal condition. Besides the probe specificity, this technique is simple, fast and low cost on sample preparation and it is

semi-quantitative for measurement. Furthermore, large numbers of sample could be detected simultaneously. Therefore, implementation of dot blot assay to detect the expression of gene contributing drought tolerance could be useful for screening task.

Besides phenotypical screening for traits contributing drought tolerance in maize population, the expression of a gene relating osmotic adjustment was also detected via dot blot hybridization with cDNA probe.

Objectives of this study

1. To develop hybridization probes that are specific to gene involving in trehalose biosynthetic pathway in maize and use the probe to screen maize samples
2. To phenotypically screen a maize breeding population.

CHAPTER 2

LITERATURE REVIEW

1. Field maize situation

Field maize (*Zea mays* L.) is an important economic crop. Ninety percent of maize yield has been used in the feed industry as a raw material. The expected total cultivating area and yield of field maize in Thailand is approximately 6.89 million rai and 4.78 million tons during the crop year 2022-2023 (Office of Agricultural Economics, 2021a). In 2021, the maize cultivation area in Thailand the most often planted in the Northern, Central, and Northeast regions, respectively. The northern region is the most cultivate estimated that 31 percent of Thailand as Nan, Tak, Chang Rai, Phare, and Payao province accounted for 9.77%, 8.30%, 5.71%, 4.18%, and 3.71%, respectively. The maize cultivation is also high in the Central region such as Phetchabun (12.75%), Nakhon Sawan (4.25%), and Phitsanulok (4.01%). The maize planted area in northeast region of Thailand was about 17.51% of the total area such as Nakhon Ratchasima and Loei provinces accounted for 10.39% and 7.12%, respectively (Office of Agricultural Economics, 2021b). Also, the value of goods exported throughout the world amounted to approximately 26.48 thousand tons, which come to approximately 256.79 million baht in year 2021 (January-October) (Office of Agricultural Economics, 2021c). However, the field maize yield slightly decreased from 4.82 million tons (2017/2018) to 4.78 million tons (2021/2022), which decreased by 0.7% per year due to drought and fall armyworm infestation (Office of Agricultural Economics, 2021a). In year 2019, the World Resources Institute (WRI) reported that Thailand was 45th of the international ranking for a shortage of water due to drought stress. (Rutger *et al*., 2019). Thailand has been being affected irregular rainfall since 2012 due to climate change and it affects agricultural production. However, the drought is continuing to affect in Thailand due to rainfall prediction have dipped below normal. (Water Crisis Prevention Center, 2021).

When maize is under water deficit conditions, morphology characteristics are defected and obviously changed. A long-term shortage of water could cause smaller leaf size, shorter plant height causing by a shorter internode, larger gap of anthesis-silking interval (ASI), lower grain yield by 15 – 25% (Muhammad *et al*., 2015; Nesmith and Ritchie, 1992). For root traits, the length and quantity of maize roots increased since maize need to deepen their roots to find water in the deeper soil for their survival. A shortage of water around the time of pollination results in the malformed embryos and abortion because of inhibition of sucrose transport in starch synthesis of seeds. As a result, grain filling and grain set are being affected (Zinselmeier *et al*., 1999) and resulting in a yield reduction of 50% (Denmead and Shaw, 1960).

2. Water translocation

2.1. Water status under moist soil

Water plays a crucial function in transporting nutrients and being used in metabolism activities within the plant cells. During the process of transpiration, the water is released into the atmosphere as vapor via stomata. At the same time, the water molecule is pulled through the xylem in roots and traveled through the stem to the leaves. The stomata open once there is enough water within the leaves, resulting in an increase in CO_2 absorption for photosynthesis. Water transportation from root to leave occurs via a balance of water potential (Ψ) between plant root and soil. Then water molecules move from cell to cell via the apoplast and symplast. Consequently, water transports to various areas for further use in the plant metabolism.

2.2. Changes of water status under water stress

Normally, plants are unable to absorb water and transport it via the stem if there is insufficient water around the roots. Furthermore, water tends to flow out of

the cell, and turgor pressure within cell drop rapidly (Nieves-Cordones *et al*., 2019). It has been discovered that a process of cell enlargement, cell wall production, and starch synthesis, are slightly affected due to a reduction of water potential in soil and leave, that is lower than -1.5 MPa. Meanwhile, stomatal conductance is lower. Transpiration and stomatal conductance are reduced as a result of this decrease. Leaf cell expansion is reduced when cell enlargement is limited. Also, when turgor pressure is lower, the leaf area under the water deficit condition is smaller. When the stomata are closed to reduce transpiration, the rate of photosynthesis is also reduced. However, plants could detect lower water levels around the roots and signals to the leaves at a rate of one meter per minute to minimize any processes as well as close stomata before leaf water potential decreases (Giménez *et al*., 2005; Takahashi *et al*., 2020).

3. Plant response mechanisms under drought stress

There are several mechanisms that plants use to adapt or protect themselves under unfavorable conditions, especially drought stress (Larkunthod *et al*., 2015). These drought resistance mechanisms are as the following.

3.1. Avoidance

Plants with drought avoidance change their morphology when they experience abiotic stresses e.g. rolling leaf, closing stomata, changing leaf orientation and the traits of roots like, thickness, deep, density and elongation, which the main determinants of drought avoidance. These results in decreasing photosynthesis, respiration, and metabolic efficiency. Furthermore, maize leaves might produce leaf hairs and synthesized wax to coat leaves in order to lower a temperature which cause reduction of transpiration (Muhammad *et al*., 2015; Touchette *et al*., 2007; Farooq *et al*., 2012). In water-limiting environments, root architecture is changed by increasing root volume density, number of lateral roots and root length, which help them to absorb more water from deeper soil.

3.2. Escape

Drought escape is a mechanism in which plants try to complete their life cycle before facing stressful conditions. They attempt to flower earlier than usual with a shorter duration so that they are enable to complete their life-cycle prior experiencing drought event. This strategy accelerates metabolic rate resulting in a rapid cell expansion and cell division in plants. Moreover, opening of the stomata leads higher rate of gas exchange and this event promotes the efficiency of leaf photosynthesis and respiration under water stress. (Shavrukov *et al*., 2017). Water stress during the maize reproductive stage affect flowering time and seed setting, which consequently causes yield losses heavily. Ability to promptly escape stress conditions of plants could increase yield crop (Muhammad *et al*., 2015).

3.3. Tolerance

Drought tolerance is a mechanism of plants to adapt themselves in physiological and molecular levels, for example, synthesizing plant hormones and enzymes, and adjusting osmotic pressure to confer drought tolerance. Under drought stress conditions, the amount of water outside the cells is dropped and leads plants to lose water and cell shrinking. Osmotic adjustment helps to maintain osmotic potential within cell. Synthesis of compatible solutes in cytoplasm and vacuole is involved. A compatible solute is termed as osmoprotectants such as proline, glycine betaine, sugar alcohols, and trehalose that help to maintain the turgor potential. Accumulation of these substances help to maintain a structure of cell, protect enzymes and small molecules from reactive oxygen species (ROS). Compatible solutes are non-toxic to cells and non-interfere with other enzyme activities. Accumulation of these compatible solutes were founded increase under drought.

4. Trehalose sugar

4.1. Trehalose biosynthesis pathway

Trehalose is a non-reducing sugar. It could be found in many organisms such as bacteria, fungi, plants, invertebrates, etc., Trehalose is synthesized from two molecules of glucose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside). Two main enzymes in trehalose biosynthetic pathway are trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) (Iordachescu and Imai, 2008). Two steps are involved in this biosynthetic pathway. Firstly, trehalose-6-phosphate (T6P) is synthesized from Uridine Diphosphate (UDP)-glucose and glucose-6-phosphate by catalyzing of trehalose-6-phosphate synthase (TPS), and secondly trehalose-6-phosphate phosphatase (TPP) catalyzes the dephosphorylation of T6P and turn to trehalose molecule (Figure 1).

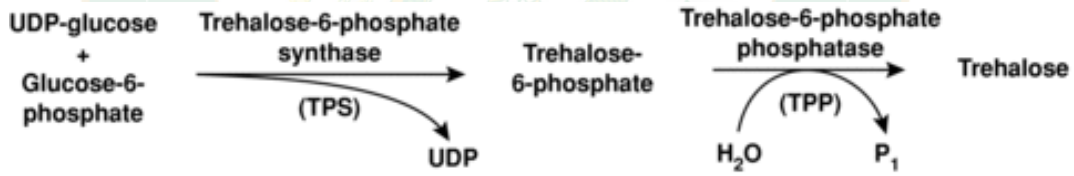

Figure 1 Trehalose biosynthesis pathway (Iordachescu and Imai, 2008)

4.2. The role of trehalose in plant

Trehalose is an important osmoprotectant and can protect other molecules from stress conditions. Trehalose sugar acts as a protectant for the stabilization of liquid bilayer during water stress (Puttikamonkol, 2015; Donnamaria *et al*., 1994; Feofilova *et al*., 2014; Pagnotta *et al*., 2010). Trehalose replaces water molecule by forming hydrogen bonds between hydroxyl groups of trehalose and phosphate of the phospholipid head group (Kumar *et al*., 2020; Lunn, 2007). Furthermore, 10-12 water molecules form hydrogen bond to one trehalose molecule (Figure 2). In drought stress, water molecules move out of the plant cell. Consequently, cell membrane structure

forms pores and the phospholipid bilayer looses as well as another protein. Then, trehalose sugar acts as an osmoprotectant to stabilize plant cell structure and led plants more tolerant to water stress (Gerszberg and Hnatuszko, 2017).

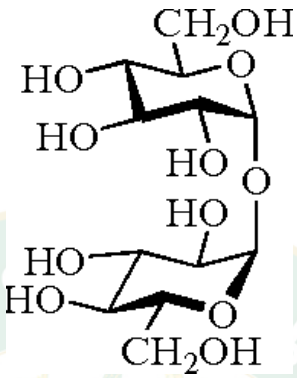

Figure 2 Structure of trehalose molecule (Feofilova *et al*., 2014)

Trehalose is found in plant cells at a very low level. However, when plants are under stressful conditions, it is found that this substance increases in concentration within cells. Trehalose replaces water molecules and bind to the cell wall with a hydrogen atom. This results in cell membrane stabilization (Puttikamonkol, 2015). Under drought stress, levels of TPS and TPP enzymes are higher. This suggests that trehalose plays an important role under drought (Grennan, 2007). Moreover, Nicolau *et al*. (2015) studied the expression of trehalose-6-phosphate phosphatase (TPP) in maize ears under drought conditions. Transgenic maize with overexpression of *ZmTPP* gene in maize ears was engineered and evaluated. The result showed yield improvement under the non-drought in range of 9% to 49%, and under severe drought conditions from 31% to 123%. This could be seen that trehalose has a role in improving plant productivity under stressful conditions.

Furthermore, Hao *et al*. (2011) developed the transgenic rice with overexpression of *OsTPS1* to enhance stress tolerance. As a result, enhancing expression level of *OsTPS1* in transgenic rice under tested drought (air-dried for 4-5

hr.). Furthermore, transgenic rice had higher survival when transferred to normal condition than wild-type rice. Comparing the results of the osmotic stress (20% PEG for 3 days) at simulates drought stress with results in increased trehalose levels, which similar to cold stress treatment (4°C for 5 days) were 78.35 and 80.68 $\mu g.g^{-1}$ fresh weight in transgenic rice lines No. 25-3-9 and 51-6-3, respectively. Under drought stress, the *OsTPS1* in transgenic rice lines expressed and consequently improved degree of tolerance. Moreover, overexpression of *AtTPS1* promoted higher accumulation of trehalose in Arabidopsis under water stress without changes in morphology (Avonce *et al*., 2004).

However, Garg *et al.* (2002) compared plant characteristics and trehalose levels between non-transgenic and transgenic rices which were subjected to drought stress (water deficit for 100 hours period for 2 cycles). Transgenic rice with overexpression *otsA* and *otsB* genes showed less wilting and rolling of young leaves than that of non-transgenic rice. Moreover, it was found that trehalose content in a shoot of non-transgenic rice (17 μg/g fresh weight) was lower than that of transgenic rice under salinity, drought, and low-temperature stress conditions. Therefore, it was likely that increasing accumulation of trehalose in transgenic rice resulted in improving degree of drought tolerance in rice.

The study of overexpression of *SoTPS1* and *SoTPS2* in drought tolerance sugarcane variety e.g., Kamphaeng Saen (KPS) varieties, which was conducted by using 16% Polyethylene glycol 6000 on the MS medium. The *SoTPS1* and *SoTPS2* expressions in Sugarcane KPS 94-13 variety were found higher than in check varieties. Moreover, the drought tolerance genotype also showed high values of leaf water potential than susceptible genotype under water stress (Saruta and Nongluk, 2020).

However, a large accumulation of trehalose is toxic or could inhibit growth in some species of plant (Liam *et al*., 2013). Several studies reported that trehalose-6-phosphate (T6P) acted as an intermediate on the trehalose biosynthesis pathway

(Grennan, 2007) and regulated molecules in plant under stressful conditions (Figueroa and Lunn, 2016). The T6P is catalyzed by Trehalose-6-phosphate synthase (TPS). Therefore, observing gene controlling *TPS* level might be useful to screen drought-tolerant plant genotypes.

4.3. Hybridization assay

Hybridization technique is a molecular tool for detecting a specific nucleic acid sequence of interest via complementary of radiolabeled or nonisotopically labeled nucleic acid probes and the target. There are many types of hybridization e.g., Southern blotting, Northern blotting and Western blotting. These three blotting techniques are used to identify regions of DNA, RNA, and protein molecule, respectively (Horn *et al*., 1986; Bhagavan and Ha, 2015). The process of hybridization technique is: (1) determining a sequence of interest, (2) developing hybridization probe that is specific to the gene, (3) performing hybridization between the developed probe and target and (4) screening or measuring of signal intensity.

Moreover, dot blot, slot blot, and colony immunoblots have been used to detect and identify molecule of interest as well. However, they are different from Southern, Northern, and Western blotting, which the samples can be aliquoted directly on membrane. There is no step of molecule separation via electrophoresis (Piazza *et al*., 2020). Dot blot and slot blot is semi-quantitative method. The advantage of these methods is that high throughput of samples could be performed at once. They are also rapid procedure, time-saving, cost-saving, and labor-saving. There are the difference between dot blot and slot blot. Dot blotting allows liquid sample dotted directly on a membrane in a single spot whereas a microtiter plate with automatic machine is used in slot blotting. Dot blot is a classic technique which is widely used since it is simple, fast and reliable. However, sensitivity and specificity of probe are the key factor for the accuracy of all hybridization techniques.

The hybridization probe is a short fragment of DNA/RNA that binds or hybridizes to gene of interest. A hybridization probe must be specific and complementary to a sequence target. A DNA probe can be generated from a DNA fragment (genomic DNA), which are highly specific to the target sequences whereas RNA probe is generated from single-stranded RNA and required a high hybridization temperature for increasing specificity and sensitivity of probe. However, RNA probe is naturally easy to be degraded by enzymes comparing with DNA probe. Therefore, RNA is often reversed to be complementary DNA (cDNA) to decrease degradation (Rishi and McManus, 1989). cDNA probe has been used to observe mRNA of interest or levels of gene expression (Litwack, 2018).

The probe is labeled with a visual indicator: radioactive and non-isotopic substances. In the past, radioactive substances for labeling were used, and the most common use was ^{32}P and ^{35}S substances. These substances should be reacted in the dark and autoradiography should be used to determine gene expression levels. Although they are substances that give explicit and rapid signals to evaluate the target gene, they are carcinogens and dangerous to researchers. As a result, non-radioactive substances is chosen to label the probe, with biotin and digoxigenin (DIG) being the most widely employed substances. These molecules (DIG or Biotin) covently bond to UTP or CTP in the DNA or RNA sequences to be labeled, and this probe will be connected to the alkaline phosphatase enzyme (AP), which will be added in the hybridization step, and then reacted with substances. The nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) are the most widely used substrates. BCIP will be dephosphorylated by AP enzyme and oxidized by NBT, resulting in the dark blue precipitate and insoluble, which causes visible (Buckingham, 2019). The excellent properties of probe hybridization should have high specificity and high sensitivity for detection.

Such a hybridization technique has been used to detect sequence targeted. There are many different methods to detect depending on the molecule of sequence targeted. The southern blot hybridization technique was introduced by Southern (1975), it is a method for studying the size and location of DNA fragments. The DNA fragments are separated based on their sizes on an agarose gel and then transferred onto a membrane for examination. When DNA has been transferred to a membrane and its fragments have hybridized to a radioactive probe, it can be visible with an autoradiograph. In the meantime, this technique has been adapted to examine sequences of RNA molecules called northern blot hybridization which was introduced by Alwine *et al.* (1977), it follows the same method as a southern blot, except it is used to detect the size of RNA or mRNA genes. It is also used to detect viruses and viroid which are infections affecting plants. Simultaneously, Hannok and Reanwarakorn (2005) used complementary DNA (cDNA) that was labeled with digoxigenin (DIG) and specifically to the viroid GYSVd-1which infects grapevine. Using the Northern blotting method, it was found that this technique can be utilized to detect infections in grape leaves with high accuracy by comparing symptomatic and non-symptomatic grape leaves of GYSVd-1 infection. Western blot hybridization is another method to detect the presence of unique proteins from mixture of protein molecules, which was introduced by Towbin *et al*. (1979). It is similar to the technique mentioned above by transferring proteins from polyacrylamide gels onto a membrane and using an antibody as a probe that is labeled with the radioactive substance. The detection of gene expression levels of protein can be observed by using both autoradiograph and UV light.

Dot-blot hybridization is another method that has a simple step, takes less time, but it very effective, rapid, and does not consider the fragment size of the target gene. It is a high-sensitivity technique for detecting gene expression levels with a small number of genes and low sample complexity, such as cloned plasmids, PCR products

and mRNA. If the gene target is complicated, the condition that the probe hybrid to the specific target gene must be optimized, especially, the temperature utilized for hybridization between probe and target gene will be determined by the length and number of nucleotides in the target gene. As a result, it must be temperature sensitive for complementary between the probe and target gene. If the condition is too severe, the probe will not attach to the gene; if it is too relaxed, the probe will attach to an unrelated gene, resulting in a false positive, which will impact the interpretation in the last step. Therefore, it is required a negative control that is free of target gene fragments and has a similar complexity for comparison and interpretation to the other samples (Buckingham, 2019). The dot-blot hybridization technique helps to save time and make the most of limited equipment, and also it is designed for screening a high throughput of samples, different species and detecting mutations in organisms. Meanwhile, the viroids of HSVd, GYSVd-1, GYSVd-2 and AGVd which cause infectious grapevine, were detected to determine gene expression levels in symptomatic and non-symptomatic grape leaves by producing specific probe of a viroid and specific polyprobe of the concurrent 4 viroids. By using the dot-blot technique, it was found that each probe that was produced can be detected viroid infections and simultaneously infected with multiple viroids (Zhang *et al*., 2012). Also, the infections of Gramineae family plants that propagate from the virus to plants through aphids can be discovered, but their symptoms are similar to those when the plants are stressed by the environment, making it difficult to identify whether the infection is caused by the environment or diseases, so the method of dot-blot hybridization by using digoxigenin probe was applied. The results suggest that this approach can detect viruses that cause diseases and separate types of them by using virus-specific probes (Liu *et al*., 2007). The aforementioned researches show that the dot-blot hybridization technique is used to detect gene expression levels and diagnose plant infections, and it also has a simple step, takes less time, requires fewer workers, and can detect numerous samples at the

same time. It can be used to determine gene expression levels related to the trehalose biosynthesis pathway under water-stressed in field maize through the use of a cDNA probe for following the genes of interest.

5. Imaging processing and analysis

Image processing and analysis is a tool to extract meaningful information from digital images, analyze important information, and calculate statistically from digital images. The imaging program is important in modern technology, also using computer programs have been used to reduce hassle and error data from being able to be visualize that helpful in providing insight diagnosing and analyzing (Ahmad *et al.,* 2021). Presently, the choice of imaging analysis program is many depending on the aim of the study. Also, phenotypic image analysis of plants is used to analyze the plant character using image data, at the same time diagnosed disease of the plant is often used the image data. The molecular biology image is often analyzed through the imaging program, which that now rarely performed completely by hand (Smith *et al.,* 2018). Evaluation of signal intensity via imaging processing and analysis improves the readability of hybridization results (Sriskanthadevan *et al.,* 2018; Ahmad *et al.,* 2021). Thus, integration of imaging software to hybridization assay could be useful for analyzing the signal intensity of probe.

CHAPTER 3

MATERIALS AND METHODS

1. Developing cDNA probes

1.1. Designing primers for Trehalose-6-phosphate synthase

To design pairs of primer that specific to gene encoding Trehalose-6-phosphate synthase (TPS), the main enzyme in trehalose biosynthetic pathway (Figure 1), the sequences of *TPS* genes for *Zea mays* L. were searched on the nucleotide database e.g., National Center for Biotechnology Information and Maize Genetics and Genomics Database. Two accession e.g., NM_001130121 (*ZmTPS*1 gene; 2,820 bp) and LOC100217143 (*ZmTPS*2 gene; 2,897 bp) were used for designing through the Primer-BLAST program. Moreover, TPS-specific primers of Nicolau *et al*. (2013) from *Saccharum officinarum* L. (*SoTPS*1) and Hao *et al*. (2011) from *Oryza sativa* L. (*OsTPS*1) were also chosen and tested along with newly designed primers. All primer sequences had been synthesized and used to amplify the *TPS* gene in maize samples.

1.2. TPS Amplification via Reverse Transcription-Polymerase Chain reaction

1.2.1. RNA extraction

Total RNA was extracted from 100 mg maize leaves by using 1 mL of TRIzol™ reagent (Thermo Fisher Scientific, USA). Each leave sample was homogenized and transferred to a 1.5 mL microcentrifuge tube and incubated in an icebox for 5 mins. Then, 200 µL of chloroform were added, vortexed, and incubated for 2-3 mins and then centrifuged at 12,000 x g for 10 mins at 4°C. The aqueous phase (400 µL) was transferred to a new-clean tube. Five hundred microliters of isopropanol were added, mixed, and incubated in an icebox for 10 mins. After that, the tube was brought to centrifuge at 12,000 x g for 10 mins at 4°C and discarded the supernatant. The pellet was washed with 1 mL of 70% ethanol and then centrifuged at 7,500 x g for 5 mins at

4°C, and kept the pellet by air-drying at room temperature for 10 mins. DNase-RNase free water (20 ul) was added to resuspend the RNA pellet. Total RNA samples were stored at -20°C until further use.

1.2.2. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Reverse Transcription reaction was carried out to synthesize single-strand cDNA. Revert Aid First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) was used for this purpose. The 20 µL RT reaction was containing 2 µL of total RNA, 2 µL of 10 pmol reverse primers, 2 µL of 10 mM dNTP mix, 4 µL of 5X RT buffer, 1 µL of RNase inhibitor (20 U/µL), 1 µL of Reverse transcriptase (200 U/µL), and 8 µL of DNase-RNase free water. Then, the reaction tube was incubated at 42°C for 60 mins in a Thermal cycler (Biometra Tone 96 G, Analytik Jena, Germany), and the reaction was terminated by heating a tube at 70°C for 5 mins, and then stored the tube at -20°C. For Polymerase chain reaction, it was performed in 25 µL reactions which contained 0.5 µL of 10 mM dNTPs, 2.5 µL of 10X PCR buffer, 0.5 µL of 10 pmol Forward Primer, 0.5 µL of 10 pmol Reverse Primer, 0.125 µL of Taq DNA polymerase (5 U/µL) (GeneDireX, Inc.), and 18.875 µL of DNase-RNase free water. PCR profile was run in Thermal cycler as the following: 94°C for 5 mins, 94°C for 40 sec, 50-58°C for 30 sec, 72 for 2 mins, and 72°C for 5 mins (30 cycles).

The published primers (Hao_*OsTPS*1) for the *TPS* gene were performed for PCR cycle steps by following PCR profile as suggested in Hao *et al*. (2011). PCR profile was 94°C for 3 mins, 94°C for 30 sec, 50-58°C for 30 sec, 72°C for 30 sec and 72°C for 7 mins (30 cycles). For the PCR profiles of Nicolau_*SoTPS1* primer (Nicolau *et al*., 2013) were 95°C for 2 mins, 95°C for 45 sec, 48-55°C for 45 sec, 72°C for 45 sec, and 72°C for 5 mins (30 cycles).

For a step of gel electrophoresis, PCR products were separated in 1% TBE agarose gel electrophoresis at 100 V for 40 mins and visualized under UV-

transilluminator (GeneFlash, Syngene USA). A stock solution of 10X TBE (Tris-borate-EDTA) buffer was prepared by mixing 100 g of Tris base, 55 g of Boric acid and 40 mL of 0.5 M EDTA (pH 8.0) in 1 L of dH_2O. For gel running buffer, 100 ml of 10X TBE buffer was added in 900 ml dH_2O to make 1X TBE working solution.

1.2.3. Gel purification for DNA sequencing

To ensure whether amplified PCR fragments belonged to the TPS region, they were sequenced (Macrogen Asia Pacific Pte Ltd., Singapore) and aligned to test their homology. To do so, amplified PCR fragments from each pair of primer were purified by using PCR Clean-Up & Gel Extraction Kit (Bio-Helix, Taiwan). According to the standard protocol of a company, 300 ul of 1% agarose gel with the desired DNA band was excised and transferred into a 1.5 mL microcentrifuge tube. A 500 µl of buffer B was added to the tube, vortexed, and incubated at 60°C for 10 mins or until the gel slice has completely dissolved. During the incubation, mixed vigorously by vortexing the tube every 2-3 mins. The dissolved sample was let stand at an ambient temperature until cool down. The supernatant was pipetted to a PG column, centrifuged at 14,000 x g for 30 seconds, and discarded the flow-through supernatant. Four hundred microliters of the Buffer W1 were added to the column PG for washing, centrifuged at 14,000 x g for 30 seconds, discarded the flow-through supernatant again. The second round of washing was carried out with 600 µl of the Buffer W2 and followed the step of the first wash. After that, the PG column was centrifuged at 14,000 x g for 2 seconds 2 times to remove the residual Buffer W2. To elute the DNA, placed the PG column in a clean 1.5 ml microcentrifuge tube and added 50 µl of the Buffer E to the center of each PG column, let it stand for 2 minutes, and centrifuged at 14,000 x g for 2 minutes to collect DNA fragment in the supernatant. This DNA sample was stored at -20°C until DNA sequencing analysis.

1.3. cDNA Probe label

TPS specific-DNA fragment with the optimal concentration (>500 ng/ul – 1 µg/ul) was labeled with DIG-11-dUTP by using DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, Germany). To do so, 16 µl of DNA solution was denatured by heating in a boiling water bath for 5 mins and quickly chilling on ice for 5 mins. Then, added 4 µL of DIG-High Prime (5X conc. labeling mixture containing the optimal concentrations of random primers, nucleotides, DIG-dUTP (alkali-labile), Klenow enzyme, and buffer components) to the denatured DNA, mixed and centrifuged briefly. The reaction mixture was incubated at 37°C, overnight. After that, added 2 µL of 0.2 M EDTA (pH 8.0) to stop the reaction and tested for their limit of detection to evaluate their efficiency.

1.4. The efficiency of labeled cDNA probe

DIG-labeled cDNA probes and DIG-labeled control DNA (5 µg/mL linearized DNA) were diluted to 1 ng/µL, and the dilution series for DIG-labeled cDNA probes and DIG-labeled control DNA was separately performed in a range of 0 - 1000 pg/µL as shown in Figure 3 e.g., 1000, 100, 50, 30, 10, 3, 1, 0.3, and 0 pg/µL. Each of the 9 serial dilutions was obtained. In addition, the 50, 30, 3 and 0.3 pg/µL dilution concentrations were added to a 10-fold serial dilution to increase resolution and observe efficiency of each probe thoroughly. The DIG-labeled control DNA and DIG-labeled probe included PH_*ZmTPS1-1*, PH_*ZmTPS1-2*, Hao_*OsTPS1* and Nicolau_*SoTPS1* probe were prepared for all 9 concentration as described above.

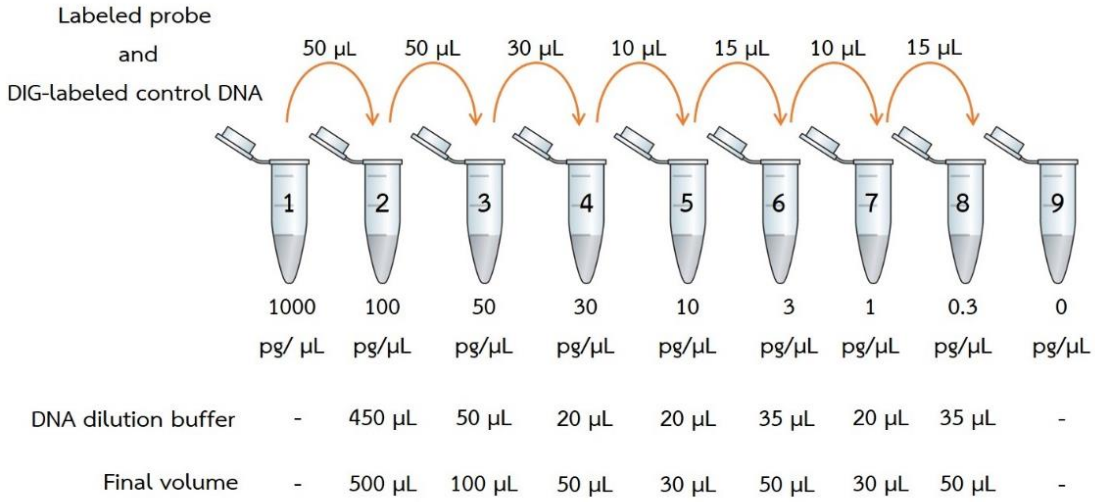

Figure 3 Prepare a dilution series of the labeled probe and DIG-labeled control DNA.

According to a company's standard protocol, aliquoted 3 µL of each diluted solution (tubes 1-9) from both DIG-labeled cDNA probes and DIG-labeled control DNA to the nylon membrane that immersed in 10X SSC (0.15 M Sodium citrate, 1.5 M NaCl, pH 7.0) for 10 mins and air-dry for 10 - 15 mins in advance. After each tube has been spotted to the membrane and air-dry for 5 minutes, DNA in membrane was fixed by cross-linking with UV light for 3 mins. Then transferred the membrane into a plastic container with 20 ml of Maleic acid buffer (0.1 M Maleic acid, 0.15 M NaCl, pH 7.5), incubated and shaked at 50 rpm on the shaker for 2 mins at room temperature, then discarded solution. Added 10 mL of 1X Blocking solution (prepared from 10X Blocking solution (Roche, Germany) at a ratio of 1:10 Maleic acid buffer) and incubated for 30 mins then discarded solution.

Prepared antibody solution by centrifuged Anti-Digoxigenin-AP at 10,000 rpm for 5 mins at 4°C, then pipetted aqueous phase from the surface and diluted the Anti-Digoxigenin-AP in 1X blocking solution at ratio of 1:5000 (150 mU/ml). Then added 10 mL of 150mU/ml Antibody solution into the membrane and shaked at 50 rpm on the shaker for 30 mins and then discarded the solution. After that, washed the membrane in 10 mL of Washing buffer (0.1 M Maleic acid, 0.15 M NaCl, pH 7.5 and 0.3% (v/v)

Tween 20) for 15 mins on a shaker, repeated 2 times. Discarded the solution and added 10 mL of Detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5), then incubate and shaked at 50 rpm for 3 mins.

To detect the probe signal, prepared color-substrate solution by adding 40 µl of NBT/BCIP stock solution in 2 ml of detection buffer, kept it away from light. Transferred the membrane to a plastic bag, then added 2 mL of color-substrate solution into a plastic bag, sealed it tightly, incubated in the dark chamber at room temperature or 37°C for 30 mins to 1 hour or until color developed. It is important not to shake the solution in this step. To stop the reaction, added 50 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and shaked for 5 mins on the shaker, then air-dried the membrane. Image was taken for the image analysis.

1.5. Hybridization with cDNA probe

The appropriate hybridization temperature was calculated based on GC content within probe sequence and percent homology of the probe to the target. The equation was showing below (Roche, Germany):

$$T_m = 49.82 + 0.41\,(\%GC) - (600/l)$$

$$T_{opt.} = T_m - 20 \text{ to } 25^{\circ}C$$

Where T_m is a melting temperature, %GC is the percentage of GC content in probe and l is the length of probe, while $T_{opt.}$ is the actual hybridization temperature for hybridization.

First step of hybridization, prepared the Nylon membrane by immersing in 10X SSC (0.15 M Sodium citrate, 1.5 M NaCl, pH 7.0) for 10 mins and air-dried for 10-15 mins then pre-heated DIG Easy Hyb buffer solution (Roche, Germany) at 42°C for 30 mins.

For Prehybridization step, placed the membrane in a plastic box or plastic bag, added pre-heated DIG Easy Hyb buffer (10 mL/100 cm^2 filter) into the container and closed it tightly. Then, prehybridized for 30 mins on incubating shaker at 50 rpm, 42°C.

For Hybridization step, denatured DIG-labeled DNA probe (about 25 ng/ml) by boiling for 5 mins and rapidly cooled in an ice box. Prepared a mixture of probe-hybridization solution by adding 5 µl of denatured DIG-labeled DNA probe (30 ng/µl) into 5 mL of pre-heated DIG Easy Hyb buffer and mixed it thoroughly and avoided forming of bubbles. Then, poured off pre-hybridization solution and added probe/hybridization mixture to the membrane, incubated and shaked in a shaker at 50 rpm, 42°C for 4 hours (no longer than 20 hours for overnight incubation).

1.6. Immunological detection

For this step, washing solution needed to be prepared in advance and it should be enough for 100 cm^2 membrane. Preparation a washing solution for washing step are divided two solutions; A first wash solution (Wash solution 1) containing 2x SSC (prepared from 10X SSC stock), 0.1% SDS (prepared from 10% Sodium Dodecyl Sulfate stock (m/v)) and the second wash solution (Wash solution 2) containing 0.5x SSC, 0.1% SDS. The wash buffer 2 are prewarmed to the temperature at 68°C before using them in the washing steps.

The post-hybridization membrane and discarded the solution, then transferred the membrane into the plastic box. Then washed the membrane with Wash solution 1 for 5 mins, twice on a shaker at room temperature, and then discarded the solution. Washed the membrane in pre-warmed Wash solution 2 at 68°C for 15 mins (twice times), discarded the solution. After hybridization, rinsed membrane briefly in Washing buffer on a shaker at 50 rpm at room temperature for 5 mins, then discarded the solution. Incubated for 30 mins in 100 ml of Blocking solution after that discarded the solution. Incubated for 30 mins in 20 ml of Antibody solution and discarded solution.

Washed the membrane in 100 mL of Washing buffer for 15 mins on a shaker, discarded the solution and washed it twice. Added 20 mL of Detection buffer, shaked for 5 mins, and discarded the solution. Then transferred the membrane to a plastic bag or suitable container and added 10 mL of color-substrate solution, closed it tight. Placed the membrane in dark at 37°C (Do not shake). The color precipitate started to develop within few minutes and the reaction usually completed after 16 hr. Stopped the reaction by washing the membrane for 5 mins with 50 mL of TE buffer on a shaker at room temperature. Image was taken for the further analysis.

2. Screening a field maize population

2.1. Testing plant materials under water stress

Completely randomized design (CRD) experimental design with 3 replications was assigned to the experimental unit. This experiment was carried out in the greenhouse during June to October 2021 at Agronomy program, Faculty of Agricultural Production, Maejo University, Chiang Mai. The 34 S_2 maize families from the previous project were screened (Hannok, 2020a). The list of maize families was shown in Table 1. The experimental unit consisted of 5 maize plants. Each maize plant was grown in a 6x15-inch growing bag (Figure 4). A total experimental unit in this experiment was 102 units (34 families * 3 reps).

Table 1 List of 34 S_2 maize families that had been used in the experiment

Code name	Origin	Code name	Origin
A1	Grp0-1-1S1	A18	Grp4-7-S1-2
A2	Grp0-1-2S1-1	A19	Grp5-2-S1-1
A3	Grp0-1-2S1-2	A20	Grp5-2-S1-2
A4	Grp0-1-2S1-3	A21	Grp2-6-1S1
A5	Grp0-3-S1	A22	Grp2-6-2S1-1
A6	Grp0-4-S1	A23	Grp2-6-2S1-2
A7	Grp0-11-1S1	A24	Grp3-3-2S1-1
A8	Grp0-11-2S1-2	A25	Grp3-3-2S1-2
A9	Grp0-11-2S1-3	A26	Grp3-5-S1-2
A10	Grp0-11-2S1-4	A27	Grp3-5-S1-3
A11	Grp0-11-2S1-5	A28	Grp3-13-1S1
A12	Grp2-7-S1	A29	Grp3-13-2S1-1
A13	Grp4-4-1S1	A30	Grp3-13-2S1-2
A14	Grp4-4-2S1-1	A31	Grp6-2-1S1
A15	Grp4-4-2S1-2	A32	Grp6-2-2S1-1
A16	Grp4-4-2S1-3	A33	Grp6-2-2S1-2
A17	Grp4-7-S1-1	A34	Grp6-2-2S1-3

Figure 4 Greenhouse experiment for assessing maize population.

Seeds were prepared by soaking in water for overnight, then transferred to the germination paper and incubated for 48 hours until radicle was emerged. Then, transferred the emerged seed into the germination tray with peatmoss for 10 days before transplanting it into the growing bag. The 30 kg/rai of compound fertilizer (15-15-15) was used as a starter fertilizer whereas 30 kg/rai of Urea fertilizer (46-0-0) or 6 g/plant was applied during 15-20 DAPs and 35-40 DAPs. Solution of either Emamectin benzoate (1.92% EC, Prochem) or Spinetoram (12% SC, Exalt) was prepared at a rate of 20 cc per 20 liters of water and were applied for preventing fall armyworm.

Water management, leave sample collection and data collection were illustrating in Figure 5 which illustrated across days after planting (DAP). Different shades of colors were displaying on a bar of DAPs. Green represented a well-watered situation and healthiness of maize plants (before water stress) whereas orange was showing a phase of ‘during stress’, in which was 7 day long (61-67 DAPs). Lastly, blue showed a

phase of ‘after stress’. Water was withhold from maize plants beginning at 53 DAP until soil moisture was dried then start counting the number of days when soil moisture is in the 1-3 dry range. Then, rewatered for recovery until the physiological maturity stage at 68 DAP. Leave samples in phases of 1) before (44 and 50 DAPs) 2) during (62, 64 and 66 DAPs) and 3) after water stress (69 DAP) were collecting for using in dot blot assay.

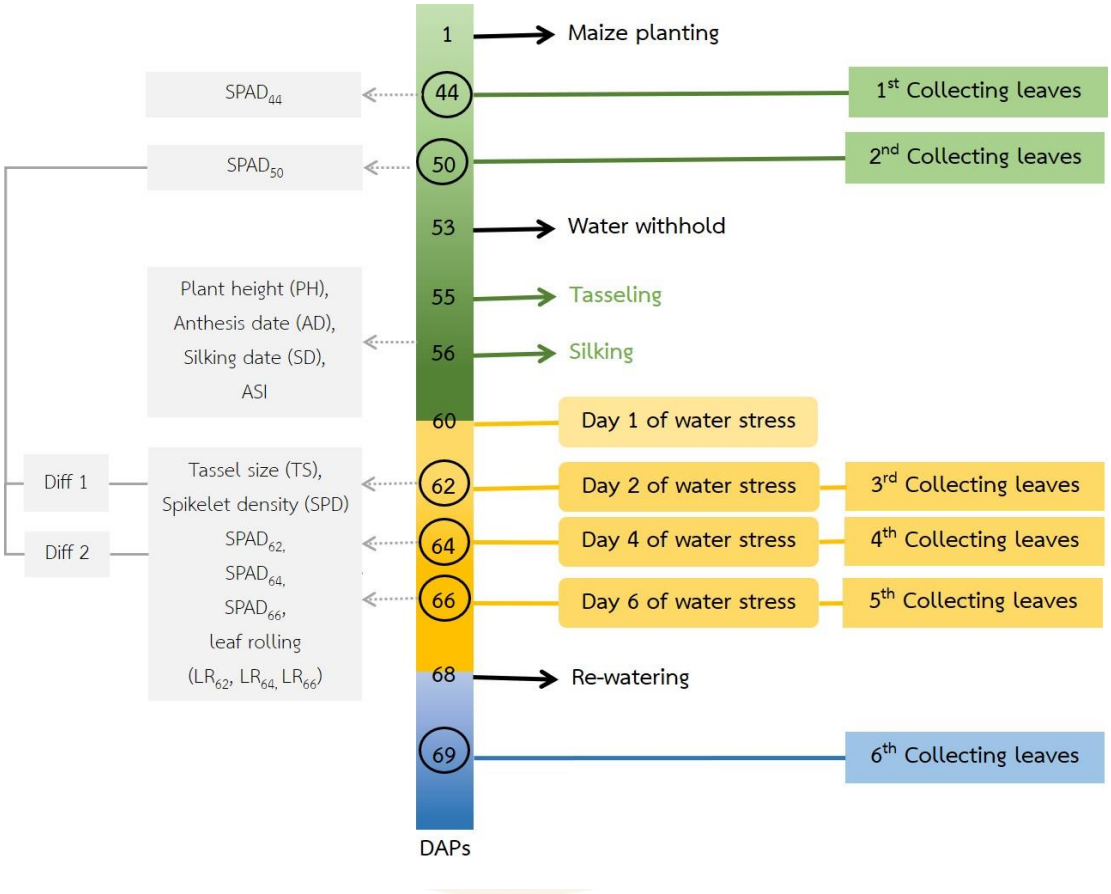

Figure 5 Experimental management at each day after planting (DAP)

2.2. Phenotyping

Six secondary traits, which recommended by CIMMYT (Bänziger *et al*., 2000) and have been using commonly in crop improvement for drought tolerance were collected in this study e.g., plant height (PH), anthesis-silking interval (ASI), leaf rolling (LR), tassel size (TS), spikelet density (SPD) and leaf greenness (SPAD). They were measured in phase of ‘before’ and ‘during’ stresses as shown in Figure 3 (left panel in

gray color boxes). An abbreviation of trait with subscription i.e., $SPAD_{50}$, LR_{62}, etc. indicated a name of trait at a specific DAP (Figure 5).

Furthermore, a change of SPAD unit while maize plants had been facing stress was also observed by calculating the differences between $SPAD_{50}$ and $SPAD_{62}$ (Diff1), and $SPAD_{64}$ (Diff2). Similarly, these Diff1 and Diff2 could suggest us which maize families were able to maintain the normal morpho-physiological traits longer over periods of stress. A method of measurement for each trait was described below

a. Plant height (PH)

Measured plant height at tasseling stage by measuring height from the soil surface to the tip of the flag leaf in centimeters.

b. Anthesis-silking interval (ASI)

Calculated ASI by subtracting days to 50% anthesis (AD) from days to 50% silking (SD)

c. Leaf rolling (LR)

During stress period, measured a circumference of rolling leaves (ear leaf) by using a measuring tape. Measured it every 2 days since water stress was given until rewatering.

d. Tassel size (TS)

Visually Scored on a scale from 1 (few branches, small tassel) to 5 (many branches, large tassel) in the flowering stage as shown in Figure 6 (Hannok, 2020b).

Figure 6 Standard score of tassel sizes on a scale from 1-5 (Hannok, 2020b)

e. Spikelet density (SPD)

Divided the main axis of tassel into three sections: apical (top of spikelet), central (center of spikelet) and basal position (bottom of spikelet) (Hannok, 2020b). Visually scored the central section by using scale 1 to 5, which 1 was less density of anthers and 5 was high density of anthers as shown in Figure 7.

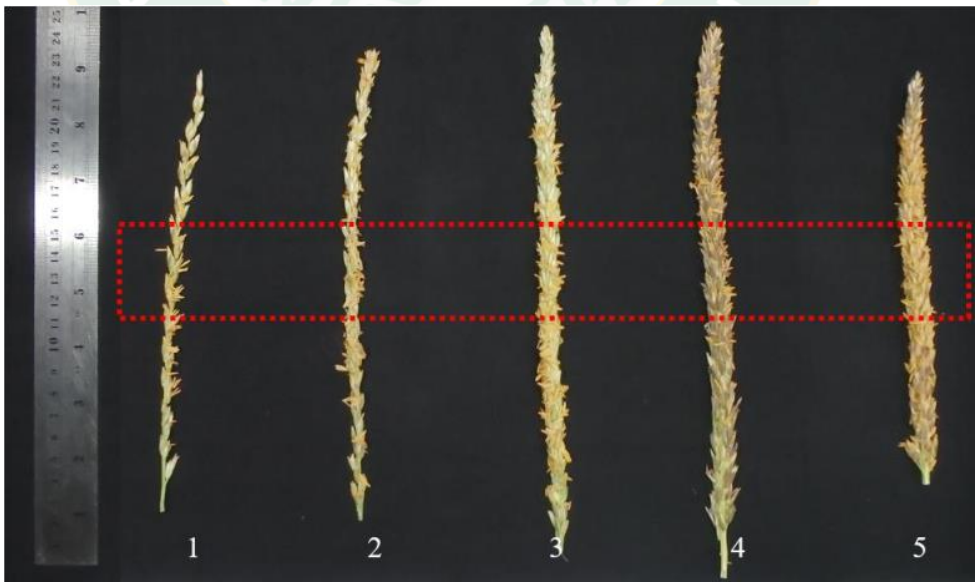

Figure 7 Standard score of spikelet density sizes on a scale from 1-5 (Hannok, 2020b)

f. Leaf greenness

Used the SPAD-502 plus chlorophyll meter (Konica Minolta, Inc.) to measure SPAD unit at 24 readings per plot.

g. Difference of leaf greenness values

The differences of leaf greenness between 'during' and 'before' stress were obtained from the equations below.

$$\text{Diff 1} = \text{SPAD}_{50} - \text{SPAD}_{62} \text{ values}$$

$$\text{Diff 2} = \text{SPAD}_{50} - \text{SPAD}_{64} \text{ values}$$

2.3. Dot blot assay

Leave samples in phases of 1) 'before stress' (44 and 50 DAPs) 2) 'during stress' (62, 64 and 66 DAPs) and 3) 'after water stress' (69 DAP) were collecting for dot blot assay. The leaves samples (100 mg) from the greenhouse test were ground with 300 µL of extraction buffer (50 mM sodium citrate, pH 8.5) in a plastic bag, transferred the extracted aqueous to a 1.5 mL microcentrifuge tube, and then centrifuged at 7,000 x g, 4^{o}C for 5 mins. Supernatant was collected and aliquoted 3 µL were spot onto nylon membrane and air-dried for 5 mins before fixing the sample by crosslinking with UV-light for 3 mins. Then a piece of membrane with samples was subjected to the steps of prehybridization, hybridization and immunological detection as previously described in the above section.

3. Determination of relative signaling intensity via imaging processing and analysis

For estimating cDNA probe sensitivity, a membrane with probe signal had been scanned in gray scale by a scanner (Canon LiDE 400, Japan) at 1200 dpi resolution. Relative signal level was measured via ImageJ program (Abramoff *et al*., 2003) and

analyzed by recommendation of Rasband (2008). A step of image analysis was described as the following.

1) Imported the grayscale image to ImageJ, inverted the gray image to black color (8-bit) on Edit menu (Figure 8A) and finally obtained 8-bit image (Figure 8B). During the inverting step, the near pixel value of the background was set to zero or as close as possible. This step increased the accuracy of the Integrated density (IntDen) and reduced noise in the background due to the intensity measurement was performed on gray area only. The IntDen values were calculated from the following formula:

$$\text{Integrated density (IntDen)} = \frac{\text{RawInden} \times \text{Area in scaled units}}{\text{Area in pixels}}$$

2) Set the parameters for the image analysis as the following: selecting Analyze >> Set measurements >> Integrated density and pressed the OK button (Figure 8C).

3) Selected the Oval selection tool, drawn the area of interest for analyzing and pressed the M (Measure) key on the keyboard to analyze (Figure 8D), then move the Oval selection through the points. The analyzed values were recorded in the Microsoft Excel, then compared the integrated intensity with the 40 ng/µl positive control for *TPS* gene in order to determine the relative intensity of the *TPS* gene.

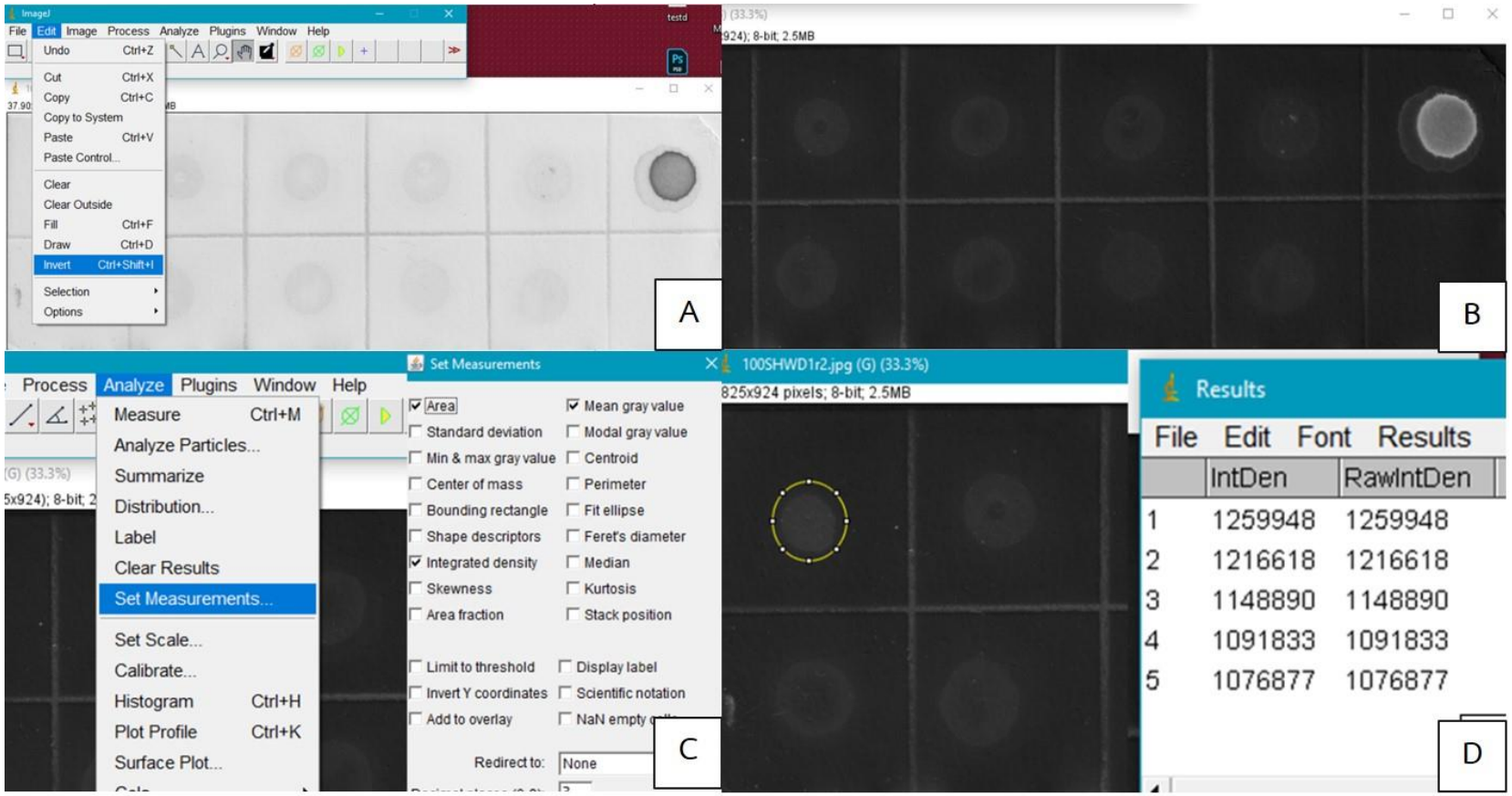

Figure 8 The analysis of signal intensity using the ImageJ program contains steps: (A) transformation of the background color, (B) black color background appearance, (C) analysis parameter settings, and (D) determination of points for analysis

4. Statistical analyses

Descriptive statistics was tested and checked for the distribution of response variables in the Microsoft Excel. Analysis of variance (ANOVA) and LSD (alpha 0.05) were performed in R statistical software V.4.0.2 (R core team, 2020). The Pearson correlation coefficients with a significance test at alpha of 0.05 were also estimated for all pairs of phenotypic traits including the relative signal intensities at all 6 different DAPs.

Moreover, Smith selection index (Smith, 1936) or Linear phenotypic selection index (LPSI) was estimated and used for ranking maize families based on multi-phenotypic traits. With the concept of unequal importance of traits for selection, Smith selection index (*I*) includes weight for each trait as seen in the following (Smith, 1936; Céron-Rojas *et al.,* 2018):

$$I = \sum_{i=1}^{t} w_i h_i^2 y_i$$

where w is the weight for *i* trait, h^2 is the narrow sense heritability for *i* trait and y is the observable value for *i* trait. To estimate the selection index which is based on multi-phenotypic traits, 3 phenotypic traits (Diff1, Diff2 and LR_{62}) had been chosen and subjected in RIndSel software (Ángela *et al*., 2017). To find the best and worst families based on Smith index, 10% cut-off was determined and maize families from both tails were considered as the most tolerant and susceptible to water stress.

CHAPTER 4
RESULTS AND DISCUSSION

1. Development of cDNA probes

1.1. Information and primers designed of *TPS* gene in field maize

Pairs of specific primer were designed for *TPS* (Trehalose-6-phosphate synthase) and *TPP* (Trehalose-6-phosphate phosphatase) genes. MaizeGDB database showed a list of 15 *ZmTPS* genes (*ZmTPS1-ZmTPS15*) and 14 *ZmTPP* genes (*ZmTPP1-ZmTPP14*). These 29 genes were analyzed in the TBtools program and found that the location of these genes was distributed on each chromosome as shown in Figure 9. *ZmTPS* genes were distributed on chromosomes 1 to 8, whereas the *ZmTPP* genes were found on chromosome 1, 2, 4, 5, 7, and 9. The length of *ZmTPS1-ZmTPS15* and *ZmTPP1-ZmTPP14* ranged from 5,184 to 14,326 bp and 3,782 to 9,141 bp, respectively (Table 2) whereas the length of the coding sequence (CDS) of *ZmTPS* and *ZmTPP* ranged from 719 to 3,377 bp and 500 to 1,325 bp, respectively (Table 2). Meanwhile, the CDS of *ZmTPS* consisted of more than 3 exons and more than 2 exons in *ZmTPP*, as shown in Table 2.

Pairs of primer were designed from the *ZmTPS1* and *ZmTPS2* genes, designed a total of 5 primer pairs. For *ZmTPS1,* coding sequence of the *ZmTPS1* gene contained 17 exons (GenBank: NM_001130121.2) as shown in Figure 10. Three specific primers for *ZmTPS1* gene accession number GRMZM2G068943 on the 8th chromosome (126,766,166 – 126,778,243 bp) were designed and named as PH_*ZmTPS1-1*, PH_*ZmTPS1-2*, and PH_*ZmTPS1-3* that designed within single genes but different locations. Each primer pair were designed to straddle between exon 3-5, exon 1-3, and exon 13-17, respectively, and fragment sizes were 370, 550 and 484 bp, respectively (Figure 10). For *ZmTPS2*, 2 primer pairs were designed from *ZmTPS2* gene accession number GRMZM2G099860, which located on chromosome 1 (28,637,045–28,644,386

bp). The mRNA template (GenBank: NM_001348792.1) consisted 3 exons. Primer pairs were named PH_*ZmTPS2-1* and PH_*ZmTPS2-2.* PH_*ZmTPS2-1* straddled between exon 1 to 2 (Figure 11) whereas PH_*ZmTPS2-2* were designed within exon 1. The expected size of fragment were 612 and 498 bp, respectively.

Moreover, pairs of primer from the published paper from Hao *et al*. (2011) and Nicolau *et al*. (2013) were used in this study and named as Hao_*OsTPS1* and Nicolau_*SoTPS1*, respectively. The Hao_*OsTPS1* was original designed from the rice chromosome (*Oryza sativa* L.) (GenBank: HM050424.1). It also found that this primer could be used to amplify the *TPS1* gene fragments of *Zea may* L. In addition, this primer bound to 5 to 17 exon regions of the *ZmTPS1* gene (GenBank: NM_001130121.2) and yielded the fragment size of 541 bp in length. The Nicolau_*SoTPS1* primer was designed from the sugarcane chromosome (*Saccharum officinarum* L.) (GenBank: EU761244.1). It also matched with maize chromosome. These two primer pairs could match with the *TPS1* gene of maize except for forward primer strands, in which only 1 nucleotide have been different (5'-TGTGCCTGTGTGTTTCTC-3'). In this study, Cytosine (C) was designed to replace Thymine (T) for increasing specificity to the *ZmTPS1* gene. More information, this primer bound to exon 6-10 of the *ZmTPS1* gene and yielded the fragment size of 400 bp. Data of all 7 pairs of primer was summarized in Table 3.

More detail about *TPS* gene was given here. The *TPS* gene in plants are generally classified into two classes: Class I and Class II. All TPS proteins in maize contain of both a TPS and TPP domain (Zhou *et al.,* 2014). In class I, *TPS* genes contain the functional gene named *ZmTPS1* which encode functional TPS enzyme and no TPP activity (Schluepmann and Paul, 2009). Moreover, *ZmTPS1* (*ZmTPS1.1*) gene shows an identical structure as same as *ZmTPS1.2* gene, which is a truncated version of *ZmTPS1.1.* Meanwhile, it is found that TPS proteins from class II (*ZmTPS*2-15) lack the first phosphatase motif which is required for the catalytic activity. Most of maize lines,

arginine (three to four amino acids) is substituted with aspartic acid at the UDPG- and G6P-binding sites (Henry *et al.,* 2014). Although, *TPS* class II has *TPS* domain but there is no TPS enzyme activity and function is still unknown (Hu *et al.,* 2020). The TPP enzyme is single-domain proteins. Which phosphatase box is conserved. It dephosphorylates T6P and produces trehalose molecule. All *TPP* genes in Arabidopsis show the unique TPP domain with conserved phosphatase domains. They encode functional TPP enzymes that have the similar activity but their expression patterns are different and specific to the tissue and growth stage (Vandesteene *et al.,* 2012). Interestingly, the *TPS1* class I is a conserve functional gene which encode TPS enzyme. Under stress conditions, this gene is up-regulated and led plants survive.

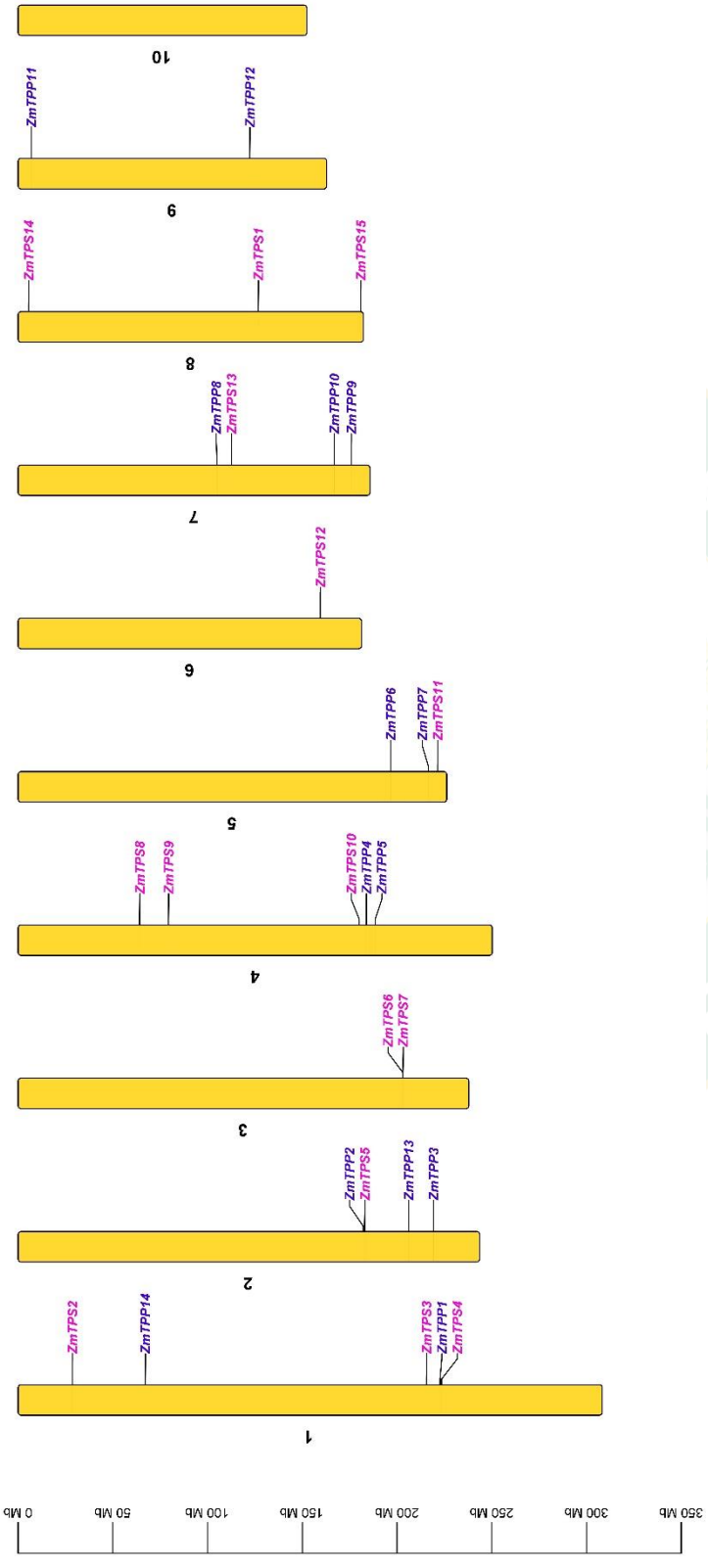

Figure 9 Chromosomes location of the genes involving trehalose biosynthesis pathway by TB tools

Table 2 Characteristics of *TPS* and *TPP* gene in *Zea mays* L.

Name	Sequence ID	Chromosome: Position (bp)	Gene length (bp)	CDS length (bp)	Exon
*ZmTPS*1	GRMZM2G068943	8: 126,766,166 - 126,778,243	12,077	2,819	17
*ZmTPS*2	GRMZM2G099860	1: 28,637,045 - 28,644,386	7,341	2,591	3
*ZmTPS*3	GRMZM2G079928	1: 215,477,373 - 215,483,857	6,484	1,943	4
*ZmTPS*4	GRMZM2G008226	1: 223,519,013 - 223,527,738	8,725	2,597	4
*ZmTPS*5	GRMZM2G527891	2: 182,934,992 - 182,944,926	9,934	2,594	5
*ZmTPS*6	GRMZM2G304274	3: 203,065,511 - 203,079,837	14,326	2,624	3
*ZmTPS*7	GRMZM2G123277	3: 203,163,097 - 203,171,021	7,924	719	3
*ZmTPS*8	GRMZM2G007736	4: 64,089,165 - 64,096,095	6,930	2,468	3
*ZmTPS*9	GRMZM2G366659	4: 79,356,171 - 79,362,976	6,805	2,606	5
*ZmTPS*10	GRMZM2G312521	4: 180,053,609 - 180,060,833	7,224	2,567	3
*ZmTPS*11	GRMZM2G122231	5: 221,423,188 - 221,430,108	6,920	2,555	3
*ZmTPS*12	GRMZM2G001304	6: 159,445,162 - 159,453,341	8,179	2,852	18
*ZmTPS*13	GRMZM2G019183	7: 112,625,667 - 112,633,186	7,519	2,666	3
*ZmTPS*14	GRMZM2G416836	8: 5,475,677 - 5,480,861	5,184	3,377	26
*ZmTPS*15	GRMZM2G118462	8: 180,916,795 - 180,925,468	8,673	2,738	3
*ZmTPP*1	GRMZM2G347280	1: 222,674,297 - 222,680,302	6,005	1,196	8
*ZmTPP*2	GRMZM2G140078	2: 182,099,411 - 182,104,953	5,542	1,154	6
*ZmTPP*3	GRMZM2G117564	2: 219,271,810 - 219,278,232	6,422	992	10
*ZmTPP*4	GRMZM2G151044	4: 183,818,385 - 183,824,400	6,015	1,070	9
*ZmTPP*5	GRMZM2G059840	4: 188,471,291 - 188,475,073	3,782	500	2
*ZmTPP*6	GRMZM2G112830	5: 196,684,422 - 196,692,031	7,609	1,109	12
*ZmTPP*7	GRMZM2G055150	5: 216,477,855 - 216,483,946	6,091	1,073	9
*ZmTPP*8	GRMZM2G174396	7: 104,975,816 - 104,981,243	5,427	1,325	4
*ZmTPP*9	GRMZM5G840145	7: 175,970,114 - 175,976,266	6,152	1,073	10
*ZmTPP*10	GRMZM2G014729	7: 175,981,197 - 175,988,707	7,510	1,085	11
*ZmTPP*11	GRMZM2G080354	9: 6,979,607 - 6,985,585	5,978	1,154	12
*ZmTPP*12	GRMZM2G178546	9: 122,330,414 - 122,338,398	7,984	1,145	11
*ZmTPP*13	GRMZM5G890599	2: 206,150,369 - 206,159,510	9,141	1,133	12
*ZmTPP*14	GRMZM6G738249	1: 67,188,898 - 67,194,249	5,351	1,094	4

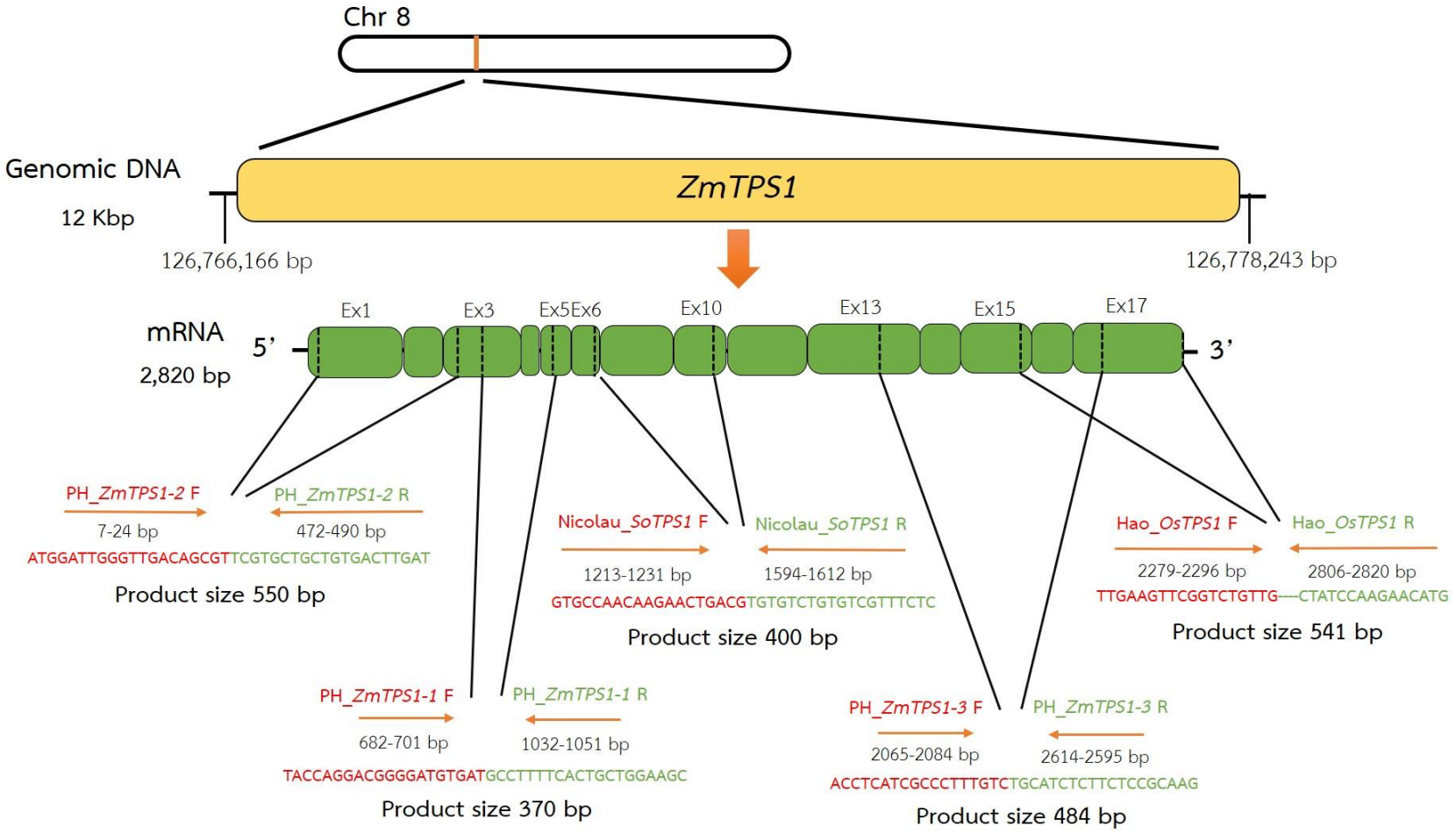

Figure 10 The mRNA structure of the *ZmTPS1* gene was used to design the primer.

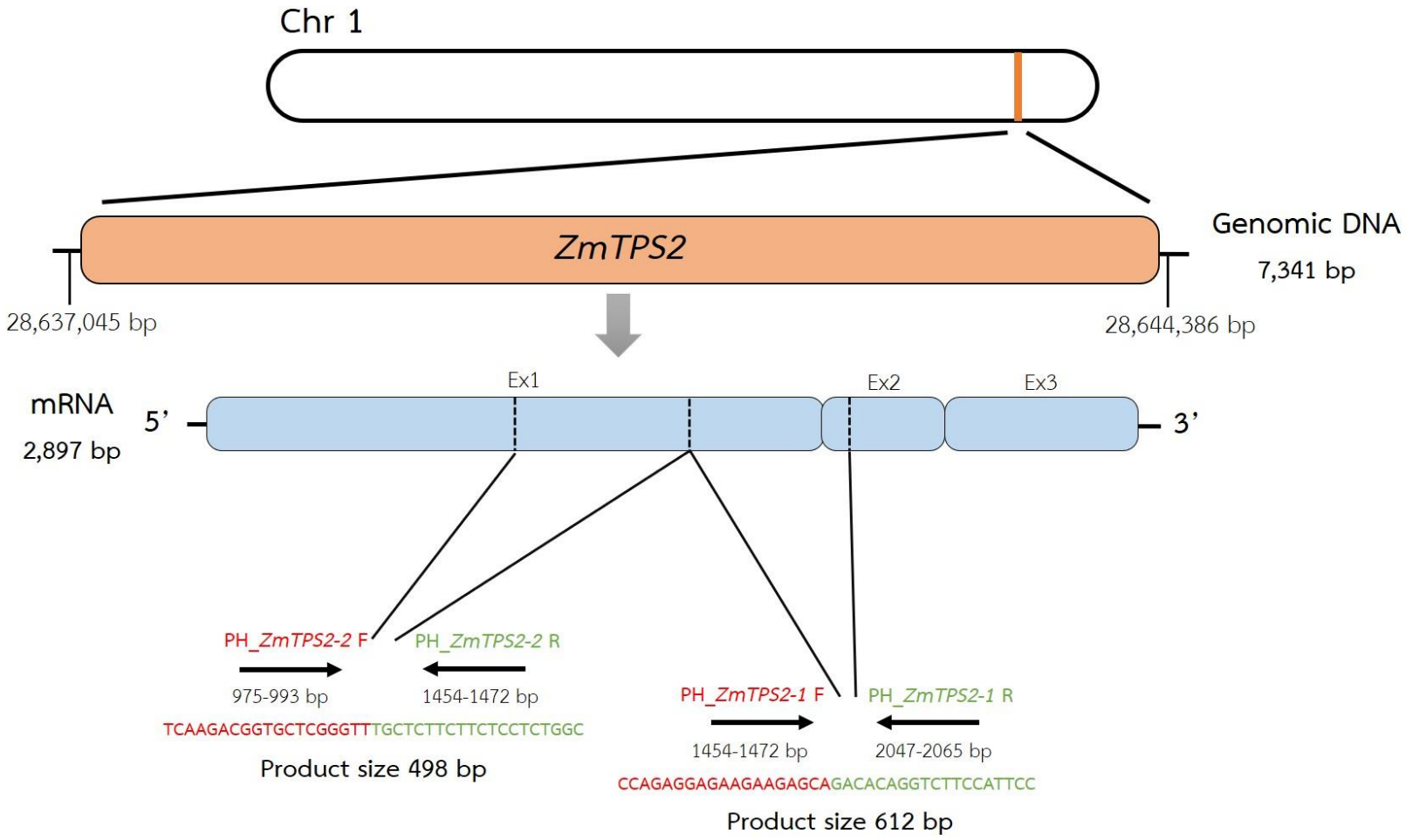

Figure 11 The mRNA structure of the *ZmTPS2* gene was used to design the primer.

1.2. Optimization of PCR conditions for specific primers to *TPS* genes

The primers were synthesized from Integrated DNA Technologies, Inc. The optimal annealing temperature for all pairs of primer was determined for PCR reaction. A annealing temperature gradient was increased by 2°C increments from calculated melting temperature (T_m) as shown in Table 3 for optimizing PCR profile and obtaining a better band intensity. For published primers, the optimal condition of Hao_*OsTPS1* and Nicolau_*SoTPS1* primers were followed as described in Hao *et al*. (2011) and Nicolau *et al*. (2013), respectively. Table 4 summarized the PCR profile for all 7 pairs of primer.

According to the results of the RT-PCR reaction, only 4 out of 7 primer pairs e.g., PH_*ZmTPS1-1*, PH_*ZmTPS1-2*, Hao_*OsTPS1* and Nicolau_*SoTPS1* yielded PCR products of expected fragment sizes. Contrastly, PCR product from PH_*ZmTPS1-3*, PH_*ZmTPS2-1*, and PH_*ZmTPS2-2* primer could not be detected. According to Figure 12, the optimal temperature for PH_*ZmTPS1-1*, PH_*ZmTPS1-2*, Hao_*OsTPS1* and Nicolau_*SoTPS1* were 50, 58, 54, and 58°C, respectively. Using these annealing temperature in PCR reaction, there was no non-specific band intensity found. Table 5 summarized the optimal PCR profile of each primer for amplifying the *ZmTPS1* gene in *Zea mays* L.

Table 3 Primer designed and characteristics.

Accession number	Primer name	Sequence (5'-3')	Nucleotides	%GC	Tm (°C)	Amplicon size (bp)
NM_001130121.2	PH_*ZmTPS1-1* F	TACCAGGACGGGGATGTGAT	20	55	60.03	370
	PH_*ZmTPS1-1* R	GCCTTTTCACTGCTGGAAGC	20	55	60.04	
NM_001130121.2	PH_*ZmTPS1-2* F	ATGGATTGGGTTGACAGCGT	20	50	59.96	550
	PH_*ZmTPS1-2* R	TCGTGCTGCTGTGACTTGAT	20	50	59.97	
NM_001130121.2	PH_*ZmTPS1-3* F	ACCTCATCGCCCTTTGTC	18	55.56	56.96	484
	PH_*ZmTPS1-3* R	TGCATCTCTTCTCCGCAAG	19	52.63	57.55	
NM_001348792.1	PH_*ZmTPS2-1* F	CCAGAGGAGAAGAAGAGCA	19	52.63	55.74	612
	PH_*ZmTPS2-1* R	GACACAGGTCTTCCATTCC	19	52.63	55.20	
NM_001348792.1	PH_*ZmTPS2-2* F	TCAAGACGGTGCTCGGGTT	19	57.89	61.50	498
	PH_*ZmTPS2-2* R	TGCTCTTCTTCTCCTCTGGC	20	55	59.10	
HM050424.1	Hao_*OsTPS1* F	TTGAAGTTCGGTCTGTTG	18	52.63	57.17	546
	Hao_*OsTPS1* R	CTGCCTATCCAAGAACATG	19	47.37	55.14	
EU761244.1	Nicolau_*SoTPS1* F	GTGCCAACAAGAACTGACG	19	44.44	52.46	400
	Nicolau_*SoTPS1* R	TGTGTCTGTGTCGTTTCTC	19	47.37	53.11	

Table 4 Conditions for testing the Annealing temperature in the PCR reaction of each primer.

Pair of primers	Temperature and Time				
	Pre-denature	30 cycles			Final Extension
		Denaturation	Annealing	Extension	
PH_*ZmTPS1-1*	5 mins at 94°C	40 s at 94°C	40 s at 50-58°C	2 min at 72°C	5 min at 72°C
PH_*ZmTPS1-2*		40 s at 94°C	40 s at 50-58°C	2 min at 72°C	5 min at 72°C
PH_*ZmTPS1-3*		40 s at 94°C	40 s at 46-52°C	2 min at 72°C	5 min at 72°C
PH_*ZmTPS2-1*		40 s at 94°C	40 s at 46-52°C	2 min at 72°C	5 min at 72°C
PH_*ZmTPS2-2*		40 s at 94°C	40 s at 48-54°C	2 min at 72°C	5 min at 72°C
Nicolau_*SoTPS1*	2 min at 95°C	45 s at 95°C	45 s at 48-55°C	45 s at 72°C	5 min at 72°C
Hao_*OsTPS1*	3 min at 94°C	30 s at 94°C	30 s at 50-58°C	30 s at 72°C	7 min at 72°C

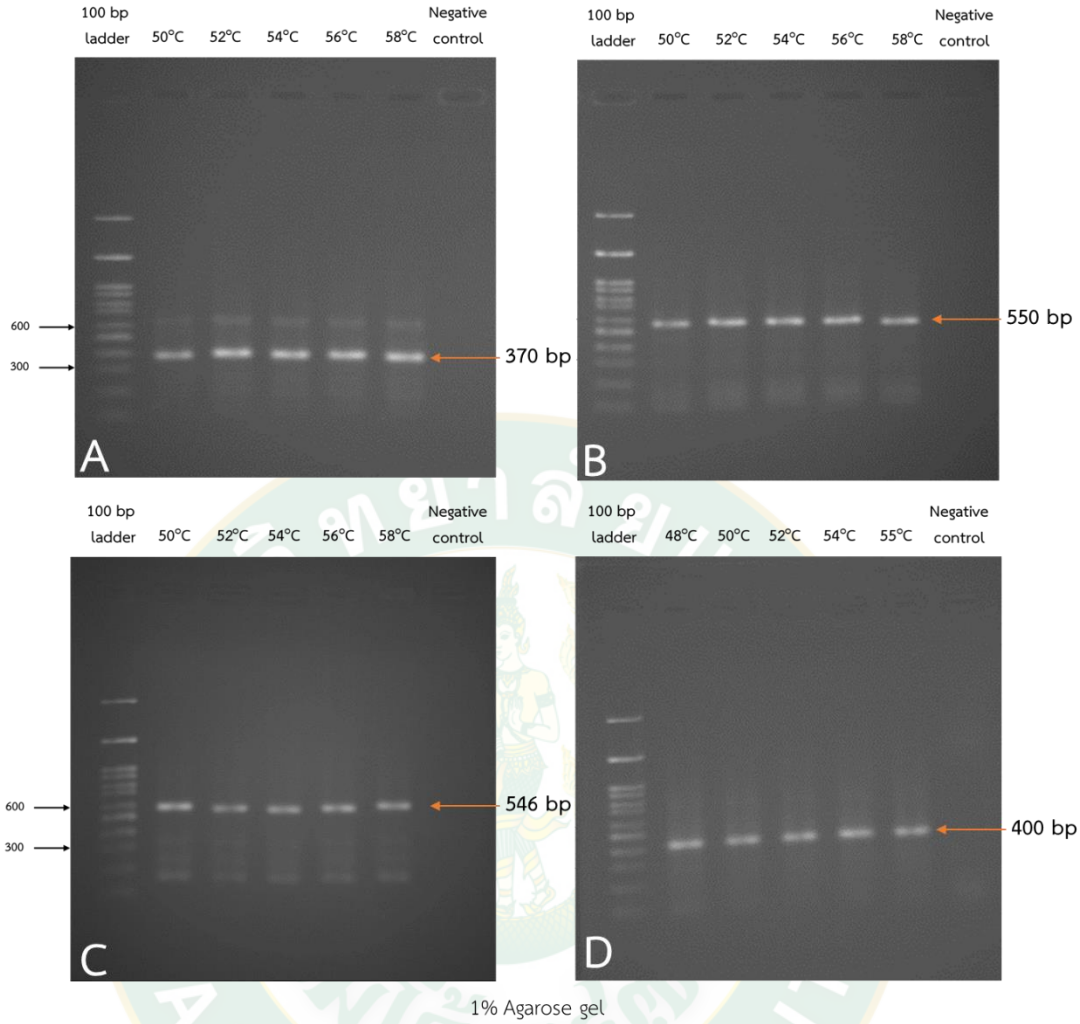

Figure 12 Comparison of different annealing temperatures in each primer.

A) PH_*ZmTPS1-1* B) PH_*ZmTPS1-2* C) Hao_*OsTPS1* and D) Nicolau_*SoTPS1*

Table 5 Optimal conditions used for amplifying the *ZmTPS1* gene in each primer.

Programs	Hao_*OsTPS1*		Nicolau_*SoTPS1*		PH_*ZmTPS1-1*		PH_*ZmTPS1*-2		Cycles
	Temp.	Time	Temp.	Time	Temp.	Time	Temp.	Time	
Pre-denature	94°C	3 mins	94°C	5 mins	94°C	5 mins	94°C	5 mins	1
Denaturation	94°C	30 sec	94°C	40 sec	94°C	40 sec	94°C	40 sec	30
Annealing	58 °C	30 sec	55°C	1 min	50°C	1 min	58°C	1 min	
Extension	72°C	30 sec	72°C	2 mins	72°C	2 mins	72°C	2 mins	
Final-extension	72°C	7 mins	72°C	5 mins	72°C	5 mins	72°C	5 mins	1

1.3. Similarity and identity of *TPS* amplified fragment from DNA sequencing

DNA sequences (Macrogen Asia Pacific Pte Ltd.) of PCR products from 4 pairs of primer (PH_*ZmTPS1-1*, PH_*ZmTPS1-2*, Hao_*OsTPS1* and Nicolau_*SoTPS1* as shown in the Appendix B) were compared to the nucleotides sequences in the NCBI database using the Nucleotide Blast tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to check the similarity to maize genome. According to the results in Table 6, PCR product of PH_*ZmTPS1-1* primer has 99% similarity to maize chromosome and was also similar to other plant species e.g., *Sorghum bicolor* (95%), *Setaria italica* (94%), *Panicum hallii* (94%), *Oryza sativa* (89%), *Brachypodium distachyon* (87%), *Triticum aestivum* (87%), *Aegilops tauschii* (87%), *Elaeis guineensis* (80%) and *Morus notabilis* (79%). Whereas PCR product from the PH_*ZmTPS1-2* primer showed 94% similarity to *TPS* of maize genome and *Saccharum officinarum* (95%), *Hordeum vulgare* (86%), *Aegilops tauschii* (85%), *Phoenix dactylifera* (85%), *Musa acuminata* (83%), *Nymphaea colorata* (84%) and *Lupinus angustifolius* (80%). Interestingly, Hao_*OsTPS1* primer, which was originally designed from rice chromosome, showed 99% similarity to maize *TPS* and *Oryza brachyantha* (a tropical grass in the Oryza genus), but not with *O. sativa.* For PCR product from Nicolau_*SoTPS1* primers, it was 95% similar to maize *TPS* (Table 6). It was interesting to observe the similarity of *TPS1* gene among species in Poaceae family. Our result corresponded to Zhou *et al*. (2013), Vicente *et al.* (2018) and Acosta-Pérez *et al*. (2020) which suggested about their homologs from different species, shown the same conserved domain of *TPS* gene.

Table 6 Comparison of similarity between maize and different species.

Primer name	Expected fragment size (bp)	Sequenced fragment size (bp)	Gene	%Identity
PH_*ZmTPS1-1*	370	400	*Zea mays* L.	99%
			Sorghum bicolor	95%
			Panicum hallii	94%
			Setaria italica	94%
			Oryza sativa	89%
PH_*ZmTPS1-2*	550	522	*Zea mays* L.	94% - 99%
			Sorghum bicolor	95%
			Saccharum spp.	96%
			Panicum hallii	92%
			Setaria italica	91%
Hao_*OsTPS1*	546	515	*Zea mays L.*	91% - 99%
			Saccharum spp.	94%
			Sorghum bicolor	92%
			Panicum hallii	89%
			Setaria italica	89%
Nicolau_*SoTPS1*	400	401	*Zea mays* L.	95% - 99%
			Saccharum spp.	96%
			Sorghum bicolor	81%
			Panicum hallii	91%
			Setaria italica	91%

1.4. The efficiency of DIG-labeled cDNA probes

PCR fragments from primer Hao_*OsTPS1*, Nicolau_*SoTPS1*, PH_*ZmTPS1-1* and PH_*ZmTPS1-2* were labelled with Digoxygenin (DIG) by using DIG High Prime DNA labeling and Detection Starter Kit. I (Roche, Germany) as described before. These 4 cDNA probes were examined for their efficiency.

According to Figure 13, signal intensity of 4 cDNA probes were compared with serial DNA labeled control. Considering a signal of Hao_*OsTPS1* probe, its intensity was greater than that of control at 3 pg/µl but less than that of control at 10 pg/µl whereas intensities from Nicolau_*SoTPS1* and PH_*ZmTPS1-2* probes had similar and they were equivalent to that of control at 10 pg/µl. Interestingly, intensity of the PH_*ZmTPS1-1* probe was equivalent to 30 pg/µl. Among 4 cDNA probes, the results suggested that the PH_*ZmTPS1-1* cDNA probe had the highest efficiency since the least detectable intensity could be found at 50 pg/ul. Therefore, PH_*ZmTPS1-1* cDNA probe was chosen for further use to ensure that an appropriate signal from the dot blot assay would be obtained for the next step of analysis.

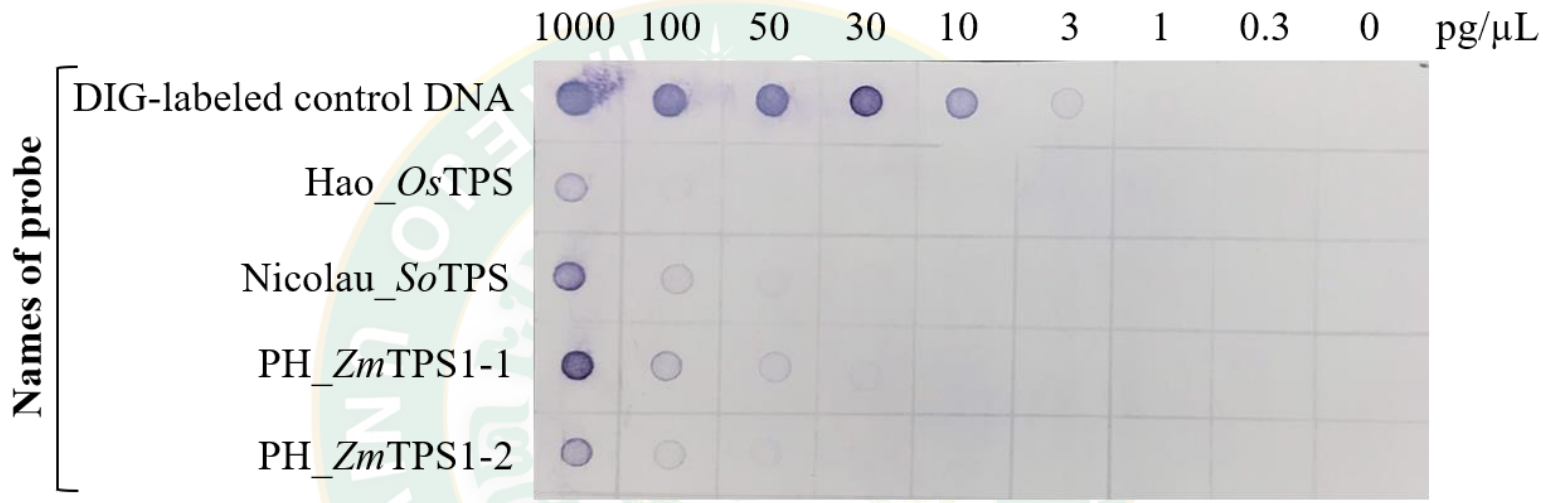

Figure 13 The efficiency of labeled cDNA probes from serial dilutions.

2. Dot blot hybridization on maize breeding population

Leaves of 34 S_2 maize families from all 3 replications grown and tested in the greenhouse were collected for Dot-blot assay at 6 different DAPs (Figure 5). Maize leaves from each plot (5 plants/plot) were bulked and weighed to 100 mg for Dot-blotting. According to the results, a Dot-blot membranes with color signal and their invert images were presented in Figure 14. RI_{44} and RI_{50} were probe signal intensity in phase of 'before' stress (44 and 55 DAP) whereas RI_{62}, RI_{64} and RI_{66} were from 'during' stress and RI_{69} was from 'after' stress. However, this study found low signal intensity in all filter membranes. Two possible explanations were 1) mRNA in dotted leave sap was low and led signal intensity was weak 2) sodium citrate extraction buffer could not help to protect mRNA and led mRNA degradation. It is well known that RNA molecule is easy to degrade.

As seen, it was difficult to visually differentiate intensity among maize samples. Therefore, a step of image processing and analysis via ImageJ was implemented to quantify the intensity. Figure 15 illustrated a plot of average signal intensity at different 6 DAPs. According to Figure 15, at 44 and 50 DAP there were 12 and 10 maize families showed *TPS* expression, in which maize family A5 was found to express *TPS* gene in both periods of 'before' stress. Furthermore, once maize families were under water stress, it was found that there were 31, 25 and 15 families expressing *TPS* at 62, 64 and 66 DAPs, respectively. It was noticed that some families continually expressed *TPS* along stress periods (62, 64 and 66 DAPs). Also, average of RI_{62} across families was highest and gradually decreased with prolonged stress periods until no signal intensity was detected at 69 DAP or 'after' stress phase (re-watering). Therefore, no bar height of RI_{69} was observed in Figure 15.

This result corresponded to studies on potato (Xu *et al*., 2017), sugarcane (Hu *et al*., 2020 and watermelon (Yuan *et al*., 2022), which reported that levels of *TPS*

expression was increased only under stress and suggested that *TPS* gene probably played a role in signal transduction pathway of stress response. Furthermore, there have been reported about the effect of overexpression of *TPS* genes in different plant species on trehalose accumulation of plants exposed to stress. They showed that seedlings of rice (Garg *et al*., 2002; Hao *et al*., 2011; Jang *et al*., 2003), Arabidopsis (Avonce *et al*., 2004) and 5 weeks-old tomato (Cortina *et al*., 2005) with overexpression of *TPS* gene showed more tolerance to stress. Also, these seedlings exhibited good phenotypes compared to wild-type seedlings. Thus, overexpression in the seedlings stage results in tolerance to stress. Moreover, it found that expression of *TPS* gene of tolerance and susceptible maize seedling occurred at 0.5 hr and 2 hr, respectively after stress (Acosta-Pérez *et al*., 2020). Similarly, the 56-day-old tolerant sugarcane had higher trehalose than susceptible one under water stress (Nicolau *et al*., 2013). In low-temperature tolerant maize, accumulation of trehalose was4.3 to 9.1-fold higher than control treatment, while low-temperature susceptible maize accumulated trehalose up to 2.5-fold of control. This increase of trehalose content played a role in low-temperature tolerance of maize (Ramazan *et al*., 2021).

Furthermore, the exogenous trehalose which applied on maize seedlings under salt and low phosphorus stress could inhibit ROS accumulation and promoted the growth of root and shoots (Rohman *et al*., 2019) and also improved the growth of sweet basil under drought stress (Zulfiqar *et al*., 2021). However, the increased trehalose showed the important role of an osmoprotectant in stress conditions. It clearly indicated that tolerant plants in seedling stage tended to highly express *TPS* gene and accumulated trehalose under many stress conditions.

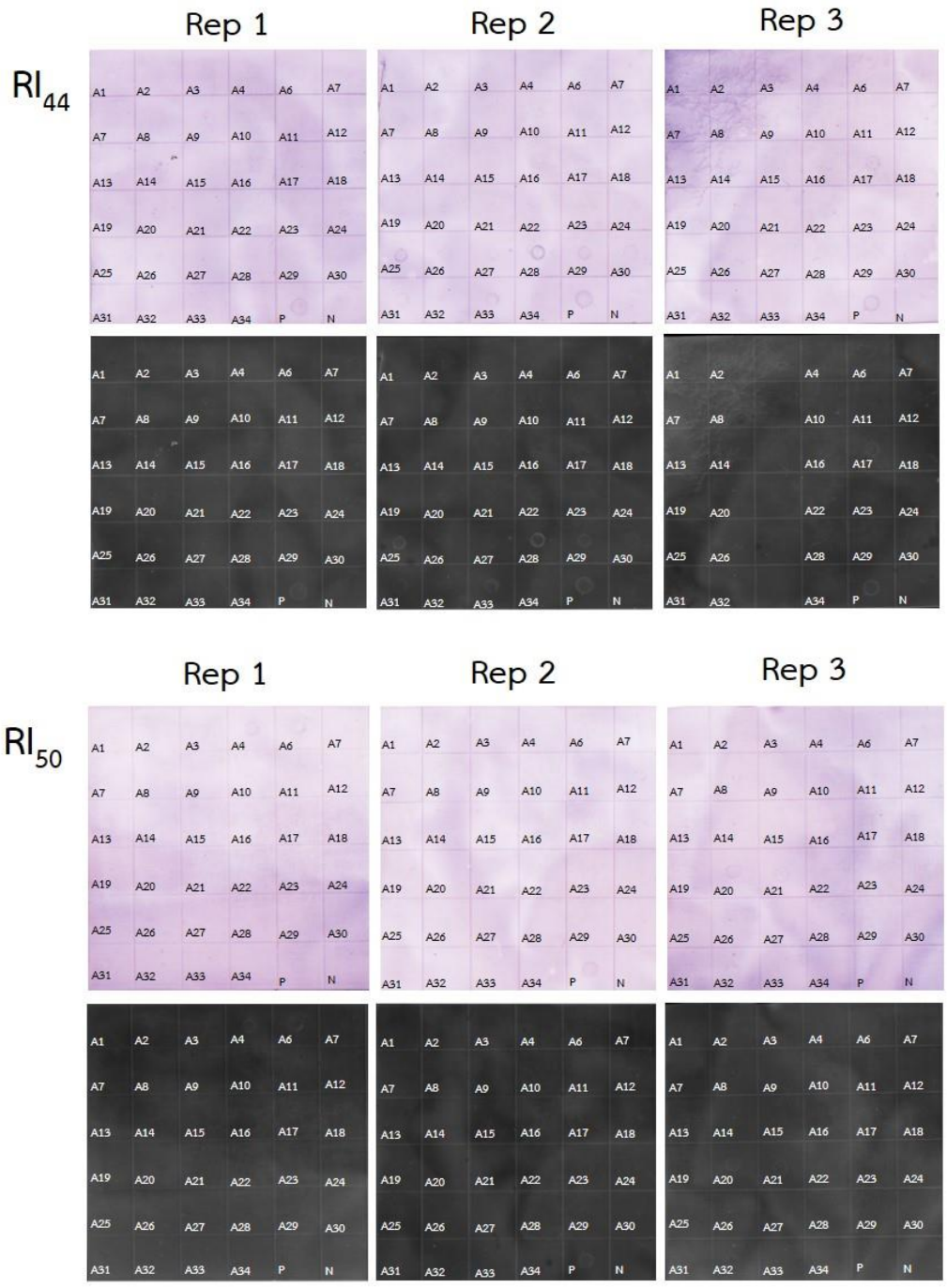

Figure 14 Relative intensities of *TPS* gene from 34 S_2 maize: relative intensities before stress (RI_{44} and RI_{50}), during stress (RI_{62}, RI_{64} and RI_{66}) of 34 maize families (A1-A34) grown and tested at 3 replications, shown as original and inverted images for imaging analysis. P is positive control (purified PCR product) and N is negative control.

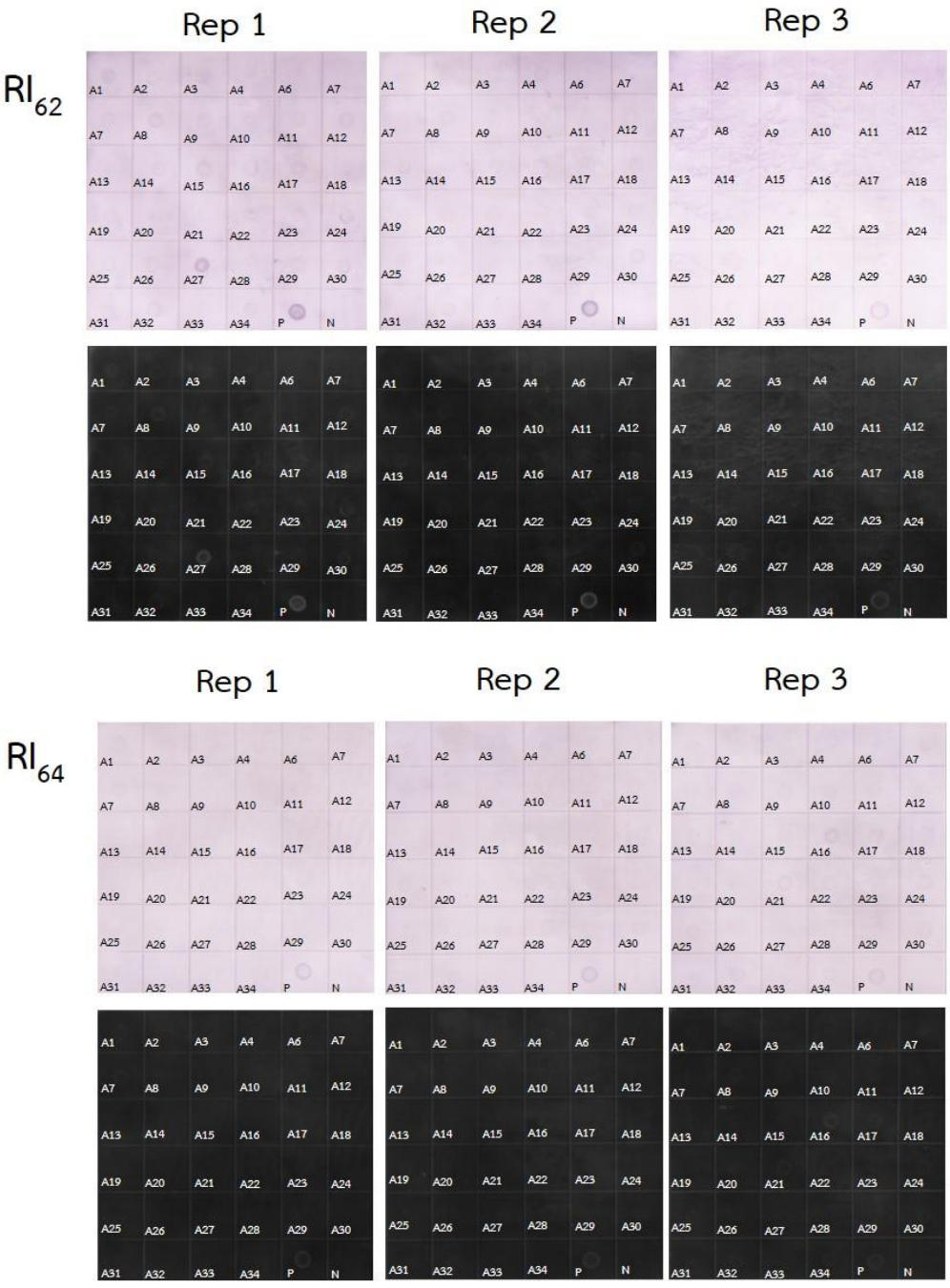

Figure 14 (Continued) Relative intensities of *TPS* gene from 34 S_2 maize: relative intensities before stress (RI_{44} and RI_{50}), during stress (RI_{62}, RI64 and RI_{66}) of 34 maize families (A1-A34) grown and tested at 3 replications, shown as original and inverted images for imaging analysis. P is positive control (purified PCR product) and N is negative control.

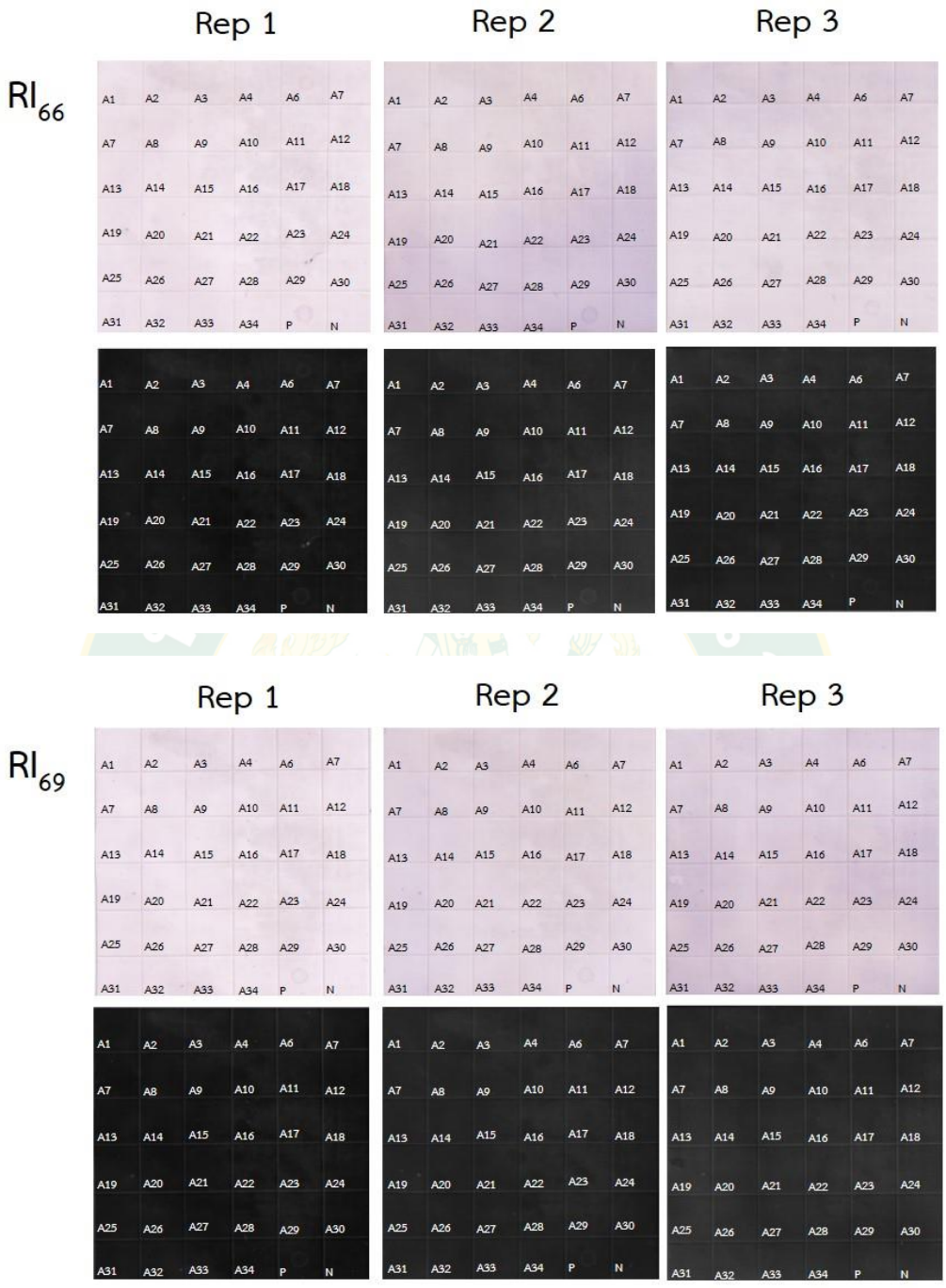

Figure 14 (Continued) Relative intensities of *TPS* gene from 34 S_2 maize: relative intensities before stress (RI_{44} and RI_{50}), during stress (RI_{62}, RI_{64} and RI_{66}) of 34 maize families (A1-A34) grown and tested at 3 replications, shown as original and inverted images for imaging analysis. P is positive control (purified PCR product) and N is negative control.

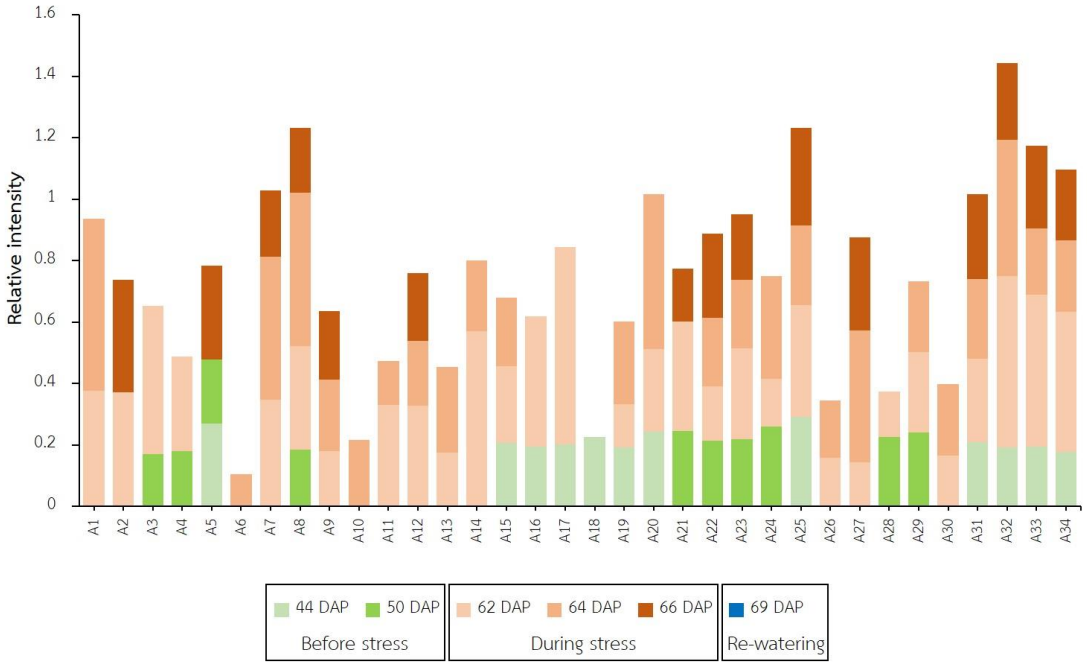

Figure 15 Average relative intensities of *TPS* gene from all 34 S_2 maize families across 6 different DAPs.

3. Phenotypic analysis and estimating Smith indices for 34 S_2 families

Sixteen characteristics collected directly from 34 S_2 maize families e.g., plant height (PH), leaf greenness (SPAD) at 44, 50, 62, 64 and 66 DAPs, leaf rolling (LR) at 62, 64 and 66 DAPs, tassel size (TS), spikelet density (SPD), day 50% to anthesis (AD), day 50% to silking (SD) and anthesis - silking interval (ASI), including differences of leaf greenness values; Diff1 and Diff2 and 5 data from image analysis e.g., RI_{44}, RI_{50}, RI_{62}, RI_{64} and RI_{66} were used for data analyses. Firstly, these data were analyzed for descriptive statistics and shown in Table 7. According to the skewness in Table 7, a distribution of 11 phenotypic traits was normal (skewness was in range of -1 to 1) except $SPAD_{62}$, $SPAD_{64}$, TS, SPD, and Diff1 showed skewed distribution with skewness values of -1.45, -1.22, -2.08, -3.38 and 1.25, respectively. Therefore, these non-normal distributions were transformed with mathematic function before testing of significance with one-way ANOVA. Square root ($x^{0.5}$; sqrt), negative reciprocal root (*-1/sqrt(x)*; nerecip) and square (x^2; sqr) functions were used for this purpose. RI_{64}sqrt, $SPAD_{62}$sqr, $SPAD_{64}$sqr, Diff1sqrt, and TSnerecip were given to these 5 transformed traits.

Result of One-way ANOVA was shown in Table 8, the result showed that all phenotypic traits of S_2 maize families were significantly diffirent ($p<0.05$) except $SPAD_{44}$, TSnererip, SD, and ASI ($P>0.05$). For $SPAD_{44}$, it was found that all 34 S_2 maize families showed no significant difference on leaf greenness, which a grand mean was 37.56 SPAD unit. It was interesting to observe that grand mean of tassel size was high (4.52 out of 5 score). This high score indicated that tassel morphology was good in size and high branching. This was because at 55 DAP, a moment of collecting data of tassel size, soil moisture was still good even it was decreasing since water was withhold at 53 DAP. So, male flowering (tassel emerging) still occurred normally. However, silk generally emerged later than tasseling. So, silking in this study was affected from a given stress (low soil moisture). Consequently, most of maize families delayed on silking and this caused a

large ASI. Most of maize families were barren and no ear development (as shown in the Appendix C). After water was withheld for 7 days (53-60 DAP), level of soil moisture was shown in dry moisture level (10-30%) based on a simple soil moisture meter. Ge *et al. (*2012) reported that different levels of field water capacity showed different magnitudes of water stress on plants. At moderate and severe water stress, field capacity might drop to 55 and 35%, respectively (Ge *et al*., 2012). Field water capacity for drought stress experiment should be measured at 30-40 cm soil depth. Zaman-Allah *et al.* (2016) suggested 35-40% field capacity and 15-20% permanent wilting point were appropriate to use in stress experiments in order to avoid fast depletion of soil moisture after imposing drought stress. One of the effects of water stress in plants is a stomata closure which caused by a reduction in water and turgor pressure of the guard cells. The stomatal closure is one mechanism of drought avoidance which respond initially to protect cell from losing water. Leaf rolling was the first sign of stressed plants and it could be observed visually (Dwyer and Stewart, 1984; Agurla *et al,* 2018). Therefore, this study used symptom of leaf rolling to indicate time of stress.

Three responsive traits; Diff1, Diff2, and LR_{62} traits were applied to Smith selection index analysis and a given economic weights for analysis was -1, -1, and +1, respectively. Smith selection indices (Table 9) for all 34 families ranged from (-16.264) to (-124.117). Once considered only 10% of Smith index distribution, S_2 families with highest (top 10%) and lowest (last 10%) index values were considered as tolerant and susceptible maize, respectively. Maize families in the top 10% were A28, A10, A16 and A6 families and last 10% were A32, A31, A23 and A22. When considering top 10% maize families, it was found that Diff1 and Diff 2 were 3.49 and 4.1 times lower than that of last 10%, respectively. Drought tolerant families were capable to maintain leaf greenness better than susceptible maize families. High values of Diff1 indicated a great change of leaf greenness that related to senescence of leave causing by chlorophyll

degradation (Gan, 2003; Chen *et al*., 2015). Our result was corresponded to Wingler *et al*. (2012) which reported that leaf of Arabidopsis with high T6P (trehalose-6-phosphate) expression levels resulted in earlier leaf senescence but leave with lower T6P resulted in delayed leaf senescence or stay-green traits. It was known that T6P is an intermediate of the trehalose biosynthesis pathway and the T6P played a role in plant development and performed functions in plants (Liam *et al*., 2013) such as flowering and embryo formation (van Dijken *et al*., 2004; Gómez *et al*., 2006; Iturriaga *et al*.,2009), stress signaling (Avonce *et al*., 2004; John *et al*., 2017), seed germination (Macovei *et al*., 2019) and regulation of carbohydrate metabolism (Ponnu *et al*., 2011; Wingler, 2002). In stress conditions, the T6P was synthesized along with the *TPS* gene in plants for adaptive to stress, however, large amounts accumulated of T6P that may be toxic and inhibit growth plant as well (Schluepmann *et al*., 2004).

Figure 16 showed relationship of RI across DAPs. top 10% and last 10% maize families were shown in colors. According to Figure 16, it was found that susceptible maize families (last 10% of Smith index) expressed *TPS* better than top 10% maize families. A red cross marks on the boxes have been representing the grand mean of the relative intensity of *TPS* gene for each period. It was noticed that last 10% families (triangle) showed higher levels of relative intensity at 62, 64 and 66 DAPs than their mean whereas top 10% S_2 families (square) showed much lower *TPS* expression during stress periods.

Table 7 Descriptive statistic of 16 phenotypic traits.

Variables	Leaf greenness					PH	Leaf rolling			TS	SPD	AD	SD	ASI	Difference leaf greenness	
	$SPAD_{44}$	$SPAD_{50}$	$SPAD_{62}$	$SPAD_{64}$	$SPAD_{66}$		LR_{62}	LR_{64}	LR_{66}						Diff1	Diff2
Mean	37.56	43.62	37.45	32.18	26.90	182.29	9.39	8.08	6.76	4.52	4.72	54.91	56.40	-3.34	6.17	10.55
Median	37.80	44.08	38.60	34.05	28.75	181.50	9.05	7.88	6.60	5.00	5.00	55.00	57.00	-3.00	4.98	9.9
Mode	40.00	45.93	34.60	35.20	31.10	181.00	7.60	5.93	5.63	5.00	5.00	54.00	55.00	-3.00	8.23	6.2
SD	3.55	4.56	7.17	7.68	9.62	27.52	1.99	1.47	1.46	0.93	0.78	3.02	1.50	1.81	7.77	7.23
Skewness	-0.20	-0.28	-1.45	-1.22	-0.76	0.44	0.70	0.41	0.70	-2.08	-3.38	0.23	0.19	-0.15	1.25	0.17
Range	17.10	24.10	44.63	45.02	50.90	137.33	9.63	6.32	7.23	4.00	4.00	10.00	6.00	9.00	41.17	46.3

Note: SPAD = leaf greenness, PH = plant height; LR = leaf rolling; TS = tassel size; SPD = spikelet density; AD = days to 50% anthesis; SD = days to 50% silking; ASI = anthesis-silking interval date, 44 and 50 = before stress; 62, 64 and 66 = during stress.

Table 8 Analysis of variance (ANOVA) of all phenotypic traits.

SOV	PH	Leaf greenness					Difference leaf greenness		Leaf rolling			TS	SPD	AD	SD	ASI
		$SPAD_{44}$	$SPAD_{50}$	$SPAD_{62}$ sqr	$SPAD_{64}$ sqr	$SPAD_{66}$	Diff1 sqrt	Diff2	LR_{62}	LR_{64}	LR_{66}	nerecip				
Families	1701.4^{***}	16.95^{ns}	33.33^{**}	$4.11x10^{5***}$	$3.85x10^{5***}$	206.56^{***}	3.86^{***}	155.75^{***}	6.79^{***}	4.29^{***}	3.97^{***}	$6.67x10^{-3ns}$	0.87^{*}	20.41^{***}	3.03^{ns}	3.72^{ns}
Residuals	299.5	10.55	14.75	$1.34x10^{5}$	$9.32x10^{4}$	37.18	1.28	28.54	2.55	1.12	1.23	$1.62x10^{-2}$	0.47	3.41	1.69	2.95
Total	2000.9	27.5	48.08	$5.45x10^{5}$	$4.79x10^{5}$	243.74	5.15	184.29	9.35	5.41	5.20	$2.28x10^{-2}$	1.34	23.83	4.72	6.67

Note: SPAD = leaf greenness, PH = plant height; LR = leaf rolling; TS = tassel size; SPD = spikelet density; AD = days to 50% anthesis; SD = days to 50% silking; ASI = anthesis-silking interval date, 44 and 50 = before stress; 62, 64 and 66 = during stress.

Table 9 Smith index and mean of Diff1, Diff2 and LR_{62} among 34 S_2 maize families

Codename	Organisms	Diff1	Diff2	LR_{62}	Smith index
A28	Grp3-13-1S1	-3.910	6.190	9.703	-16.264
A10	Grp0-11-2S1-4	2.447	8.257	6.743	-26.539
A16	Grp4-4-2S1-3	2.663	7.777	9.000	-30.122
A6	Grp0-4-S1	1.900	10.810	8.230	-31.167
A2	Grp0-1-2S1-1	2.600	11.533	7.920	-32.953
A19	Grp5-2-S1-1	1.267	12.277	9.090	-33.203
A27	Grp3-5-S1-3	-0.963	14.290	10.523	-33.723
A1	Grp0-1-1S1	8.097	4.430	7.557	-34.242
A7	Grp0-11-1S1	1.757	11.957	9.700	-34.785
A26	Grp3-5-S1-2	5.010	8.610	8.543	-35.064
A14	Grp4-4-2S1-1	1.767	14.510	8.090	-35.354
A4	Grp0-1-2S1-3	1.677	15.943	7.610	-36.190
A15	Grp4-4-2S1-2	3.913	10.647	9.667	-37.343
A9	Grp0-11-2S1-3	2.473	15.940	8.633	-39.470
A3	Grp0-1-2S1-2	4.110	14.387	8.500	-40.523
A11	Grp0-11-2S1-5	3.857	12.790	10.257	-40.928
A30	Grp3-13-2S1-2	5.290	11.423	9.767	-41.220
A5	Grp0-3-S1	6.397	12.087	8.047	-41.385
A21	Grp2-6-1S1	5.437	12.903	8.643	-41.512
A18	Grp4-7-S1-2	6.220	12.620	7.933	-41.522
A24	Grp3-3-2S1-1	3.533	15.077	10.010	-42.771
A29	Grp3-13-2S1-1	3.877	15.110	10.777	-44.771
A33	Grp6-2-2S1-2	3.823	18.200	8.843	-45.355
A13	Grp4-4-1S1	6.277	12.297	11.010	-46.352
A25	Grp3-3-2S1-2	3.857	17.423	13.323	-51.905
A8	Grp0-11-2S1-2	9.967	18.100	7.290	-54.809
A17	Grp4-7-S1-1	9.157	20.290	10.490	-61.311
A12	Grp2-7-S1	7.543	26.743	11.757	-68.397
A34	Grp6-2-2S1-3	7.677	29.910	11.790	-72.725
A20	Grp5-2-S1-2	9.510	30.677	11.557	-76.939
A32	Grp6-2-2S1-1	14.933	26.377	11.363	-81.916
A31	Grp6-2-1S1	19.077	30.877	8.933	-91.771
A23	Grp2-6-2S1-2	18.377	39.157	9.013	-101.001
A22	Grp2-6-2S1-1	30.200	38.863	9.053	-124.117

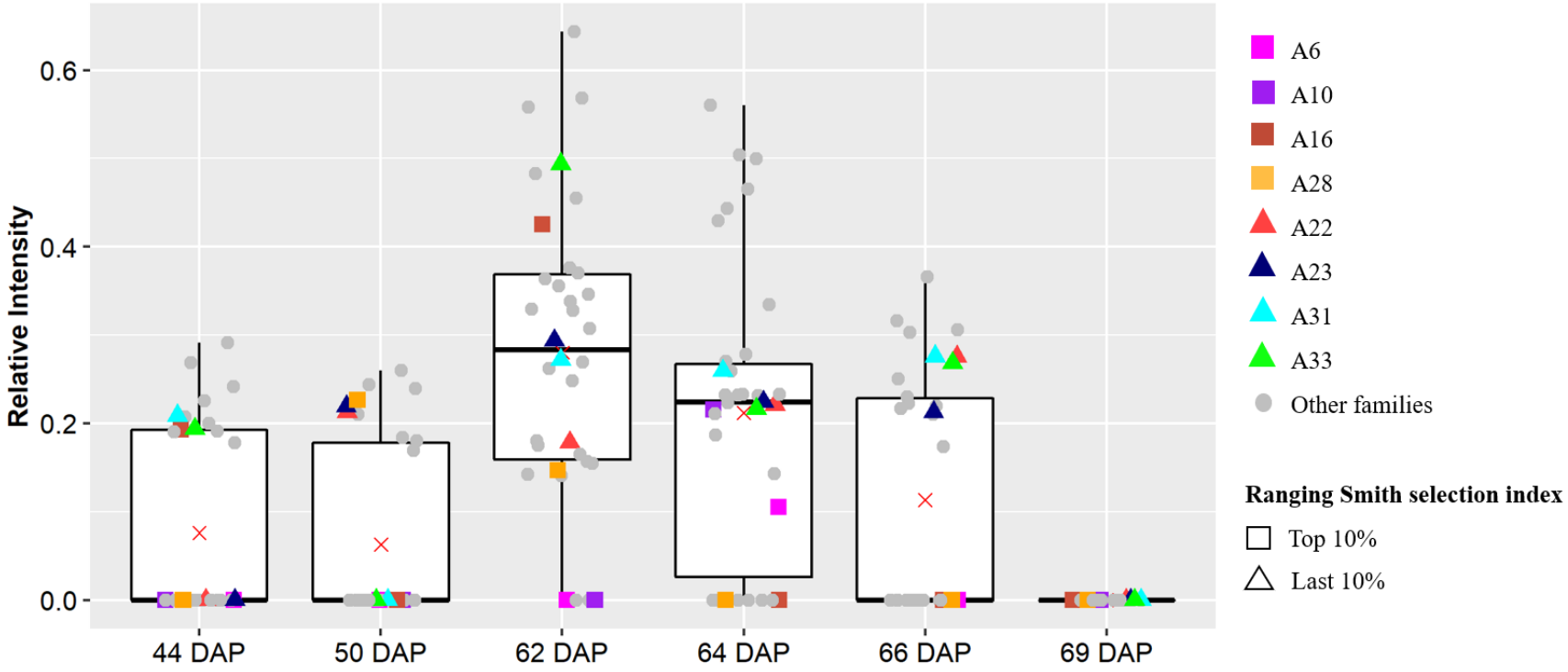

Figure 16 The levels of relative intensity of dot blot signals of 34 S_2 maize families across 6 different DAPs. Red cross mark is overall mean value of all maize families.

4. Relationship between phenotypic traits and relative signal intensity

Correlations between 16 phenotypic traits and 5 relative intensities of *TPS* gene from 5 different DAPs (no RI_{69}) were presented in Table 10. For this present study, the phenotypic traits e.g., plant height (PH), leaf greenness (SPAD), leaf rolling (LR), tassel size (TS), spikelet density (SPD) and anthesis-silking interval (ASI) were used as an indicator of drought tolerance as recommended by Hannok (2020b). Pearson correlation coefficients were in the range of -0.81 to 0.93. However, most of the relationships between traits were weak in both positive and negative directions. Strong, moderate and weak relationships with statistical significance could be found as shown in Table 10 with the bold letters.

Correlations between 6 phenotypic traits ($SPAD_{50}$, $SPAD_{62}$, $SPAD_{66}$, Diff1, Diff2 and LR_{62}) and 5 relative intensities of *TPS* gene expression at different DAPs for all 34 S_2 families under water stress were presented in Table 10. According to the Table 10, relative signal intensity at 66 DAP (RI_{66}) had a significantly negative correlation with $SPAD_{62}$ (r = -0.28, p<0.05) and $SPAD_{66}$ (r = -0.21, p<0.05) whereas positive relationship of RI_{66} was found with Diff1 (r = 0.25, p<0.05). Although only weak associations of RI_{66} were found here, but it was improved with Smith index (r = -0.42, p<0.05) as shown before. This moderate negative relationship of RI_{66} and Smith index based on Diff1, Diff2 and LR_{62} confirmed that detection of *TPS* gene expression of maize at a longer period of stress duration might be helpful to select for stay-green phenotype, which is one of the desirable traits for drought tolerance in maize. However, lower level of *TPS* expression during prolonged period of drought stress is favorable to select for. Contrast to stay-green, leaf senescence is caused by chlorophyll degradation in which many plant species go through drought stress (Chen *et al*., 2015; Gan, 2003). This eventually causes early leaf senescence and barren plants. *TPS* gene was upregulated when plants experience abiotic stresses (Schluepmann *et al*., 2004; Zhang *et al*., 2019). Furthermore, the levels of T6P (intermediate molecule in Trehalose biosynthetic

pathway) in mature plants were reported that it was higher in early senescing leaves (Wingler, 2002).

Table 10 Correlations between phenotypic traits and relative intensities of *TPS* gene

	RI_{44}	RI_{50}	RI_{62}	RI_{64}	RI_{66}	PH	$SPAD_{44}$	$SPAD_{50}$	$SPAD_{62}$	$SPAD_{64}$	$SPAD_{66}$	Diff1	Diff2	LR_{62}	LR_{64}	LR_{66}	TS	SPD	AD	SD	ASI
RI_{44}	1																				
RI_{50}	0.00	1																			
RI_{62}	-0.08	**-0.20**	1																		
RI_{64}	-0.01	0.06	-0.19	1																	
RI_{66}	-0.02	-0.02	0.17	-0.14	1																
PH	-0.05	-0.14	0.04	0.13	-0.13	1															
$SPAD_{44}$	**-0.25**	0.09	0.05	-0.05	-0.06	-0.15	1														
$SPAD_{50}$	-0.11	-0.04	0.10	0.06	-0.01	**0.32**	**0.43**	1													
$SPAD_{62}$	-0.03	-0.06	-0.08	0.03	**-0.28**	0.17	0.06	0.18	1												
$SPAD_{64}$	-0.11	-0.09	-0.08	-0.01	**-0.26**	0.18	0.11	0.13	**0.88**	1											
$SPAD_{66}$	-0.16	-0.11	-0.07	-0.03	**-0.21**	0.16	0.13	0.08	**0.66**	**0.94**	1										
Diff1	-0.03	0.03	0.13	0.00	**0.25**	0.02	0.19	**0.42**	**-0.82**	**-0.73**	**-0.56**	1									
Diff2	0.04	0.06	0.13	0.04	**0.23**	0.01	0.12	**0.42**	**-0.71**	**-0.84**	**-0.81**	**0.90**	1								
LR_{62}	0.070	-0.13	0.06	0.14	0.03	0.00	-0.04	0.07	-0.01	-0.06	-0.09	0.05	0.10	1							
LR_{64}	0.07	-0.07	0.11	0.08	0.09	0.03	-0.02	0.16	-0.15	**-0.25**	**-0.27**	**0.25**	**0.32**	**0.89**	1						
LR_{66}	0.05	0.04	0.15	-0.03	0.15	0.06	0.01	**0.24**	**-0.30**	**-0.41**	**-0.42**	**0.42**	**0.50**	**0.44**	**0.79**	1					
TS	-0.06	-0.01	0.01	0.03	-0.13	0.16	**0.32**	**0.34**	0.04	0.00	-0.02	0.15	0.18	-0.02	0.01	0.05	1				
SPD	-0.13	-0.11	0.04	0.05	-0.09	0.19	**0.34**	**0.42**	0.07	0.06	0.04	0.18	0.17	-0.01	0.04	0.10	**0.89**	1			
AD	-0.02	-0.13	0.13	0.10	-0.08	**0.22**	0.09	**0.21**	0.09	0.06	0.02	0.03	0.05	-0.02	-0.02	-0.02	**0.53**	**0.65**	1		
SD	-0.05	-0.04	0.10	0.05	-0.02	**0.38**	-0.13	**0.22**	0.17	0.15	0.11	-0.03	-0.02	-0.09	-0.06	0.00	**0.23**	**0.22**	**0.18**	1	
ASI	0.00	-0.08	-0.08	-0.06	0.03	**-0.33**	0.05	**-0.24**	-0.17	-0.16	-0.129	0.021	0.017	0.136	0.088	-0.01	**-0.24**	**-0.21**	-0.10	**-0.81**	1

Note: RI = Relative intensity of TPS gene; ASI = anthesis-silking interval date; PH = plant height; LG = leaf greenness; LR = leaf rolling; TS = tassel size; SPD = spikelet density; AD = days to 50% anthesis; SD = days to 50% silking; 44 and 50 = before stress; 62, 64 and 66 = during stress. The bold text values show statistically significant differences (p <.05).

CHAPTER 5

CONCLUSIONS

Dot blot hybridization with PH_*ZmTPS1-1* cDNA probe integrated with image analysis for detecting level of *TPS* gene expression was effective and efficient to use in screening our S_2 maize families for drought tolerance based on trehalose biosynthesis pathway. According to our results, the level of *TPS* gene expression was highest at 4 days after stress (relative intensity at 64 DAP). However, dot blotting at 6 days after stress (relative intensity at 66 DAP) was effective to differentiate maize families. Another supportive evidence was a moderate negative relationship between relative signal intensity at 66 DAP (RI_{66}) and Smith index based on multi-phenotypic traits (Diff1, Diff2 and LR_{62}) which was found to be statistically significant. Assessing *TPS* gene expression in maize at prolonged duration of stress is recommended. More importantly, our study showed that maize with high *TPS* gene expression tended to be less tolerant to water stress. It is noteworthy that *TPS* gene expression in mature maize under stress in this study showed the contrast results from the other previous reports on seedlings. Furthermore, we found that 4 out of 34 S_2 maize families with codes A6, A10, A16 and A28 based on their Smith indices might have some potentials for further use in our breeding program.

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APPENDIX A

APPENDIX A

Preparing reagents for RNA extraction

1. 70% (v/v) Ethanol 1000 mL

Absolute ethanol	700 mL
dH_2O adjust to 1000 mL	

Preparing reagents for gel electrophoresis systems

2. 10X TBE buffer (Tris-Borate-EDTA) 1000 mL

Tris base	100 g
Boric acid	55 g
0.5 M EDTA (pH 8.0)	40 mL
dH_2O adjust to 1000 mL	

3. 1X TBE buffer 1000 mL

10X TBE buffer	100 mL
dH_2O adjust to 1000 mL	

4. 1% TBE Agarose gel electrophoresis

Agarose agar	1%
1X TBE buffer adjust to final volume	

5. 0.5 M EDTA stock solution (pH 8.0) 500 mL

Ethylene diamine tetraacetic acid; EDTA	93.05 g
dH_2O adjust to 500 mL	

6. DNA loading dye buffer

6X Fluorescent loading dye (Novel juice, GeneDieX)	1 µL
PCR samples	5 µL

7. DNA ladder

100 bp DNA ladder RTU (One mark 100, GeneDieX)	2 µL
6X Fluorescent loading dye (Novel juice, GeneDieX)	1 µL

Preparing reagents for Dot blot hybridization assay

8. 0.2 M EDTA (pH 8.0) 500 mL

0.5 M EDTA stock solution	200 mL
dH_2O	300 mL

9. 1X Maleic acid buffer (pH 7.5) 1000 mL

0.1 M Maleic acid	11.6 g
0.15 M NaCl	8.8 g
Adjust pH to 7.5 with NaOH (Solid)	
dH_2O adjust to 1000 mL	

10. Washing buffer 1000 mL

0.1 M Maleic acid	11.6 g
0.15 M NaCl	8.8 g
0.3% Tween 20 (v/v)	3 mL
Adjust pH to 7.5 with NaOH (Solid)	
dH_2O adjust to 1000 mL	

11. Detection buffer 1000 mL

0.1 M Tris base	12.114 g
0.1 M NaCl	5.844 g
Adjust pH to 9.5 with HCl	
dH_2O adjust to 1000 mL	

12. 1X Blocking solution 25 mL/100 cm^2

10X Blocking solution	2.5 mL
1X Maleic acid buffer	22.5 mL

13. Antibody solution 10 mL/100 cm^2

Antibody solution (150 mU/ml)	2 µL
1X Blocking solution	10 mL

14. TE buffer 1000 mL

0.01 M Tris base	1.2117 g
0.001 M EDTA	0.29224 g
Adjust pH to 8.0 with HCl	
dH_2O adjust to 1000 mL	

15. 10X SSC stock solution 1000 mL

0.15 M Saline sodium citrate	38.709 g
1.5 M NaCl	87.66 g
Adjust pH to 7.0 with HCl	
dH_2O adjust to 1000 mL	

16. 2X SSC stock solution 500 mL

10X SSC stock solution	100 mL
dH_2O	400 mL

17. 0.5X SSC stock solution 500 mL

10X SSC stock solution	25 mL
dH2O	475 mL

18. 10% SDS Stock (m/v) 500 mL

Sodium dodecyl sulfate	50 g
dH2O adjust to 500 mL	

19. 0.1% SDS Stock (m/v) 500 mL

10% SDS Stock	5 mL
dH2O	495 mL

20. Color substrate solution 2 mL/100 cm^2

NBT/BCIP solution	4 µL
Detection buffer	2 mL

Preparing reagents for extraction buffer

21. 100 mM Sodium Citrate (pH 8.5) 200 mL

Sodium Citrate Dihydrate	5.882 g
dH_2O adjust to 200 mL	

22. 50 mM Sodium Citrate (pH 8.5) 100 mL

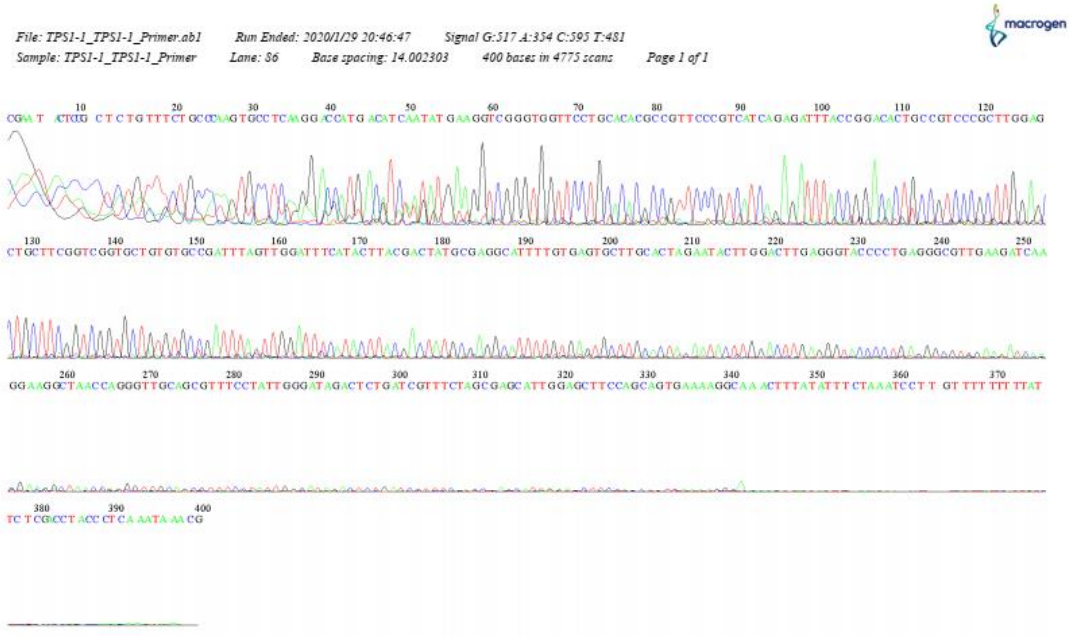
100 mM Sodium Citrate	50 mL
dH_2O	50 mL

APPENDIX B

APPENDIX B

DNA sequencing from gel extraction PCR product of *TPS* gene in Ki11 maize variety

>PH_*ZmTPS1-1* primer



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TGGGATAGACTCTGATCGTTTCTAGCGAGCATTGGAGCTTCCAGCAGTGAAAAGGCA
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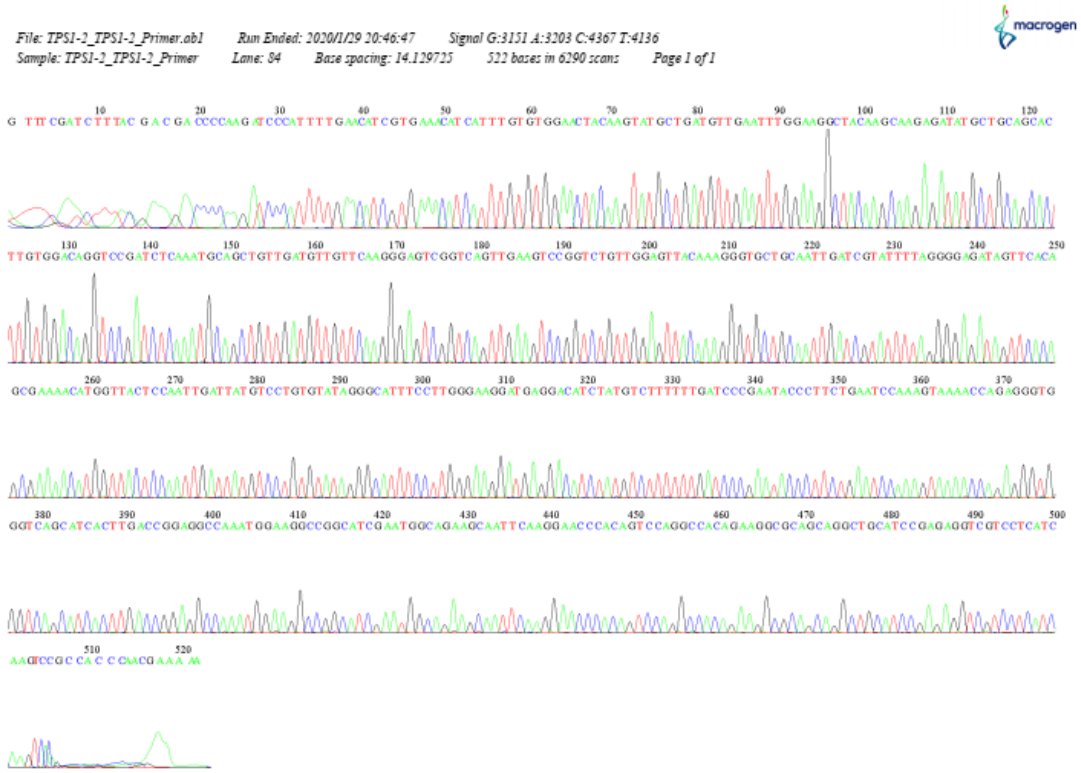
G

>PH_*ZmTPS1-2* primer



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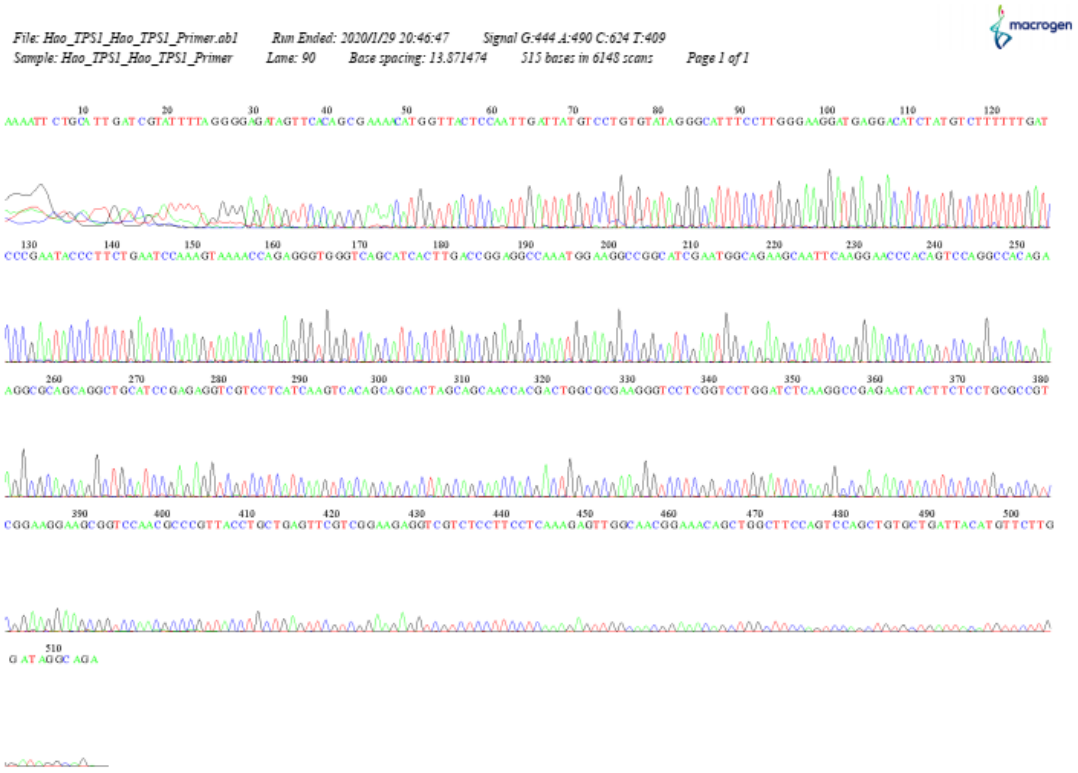

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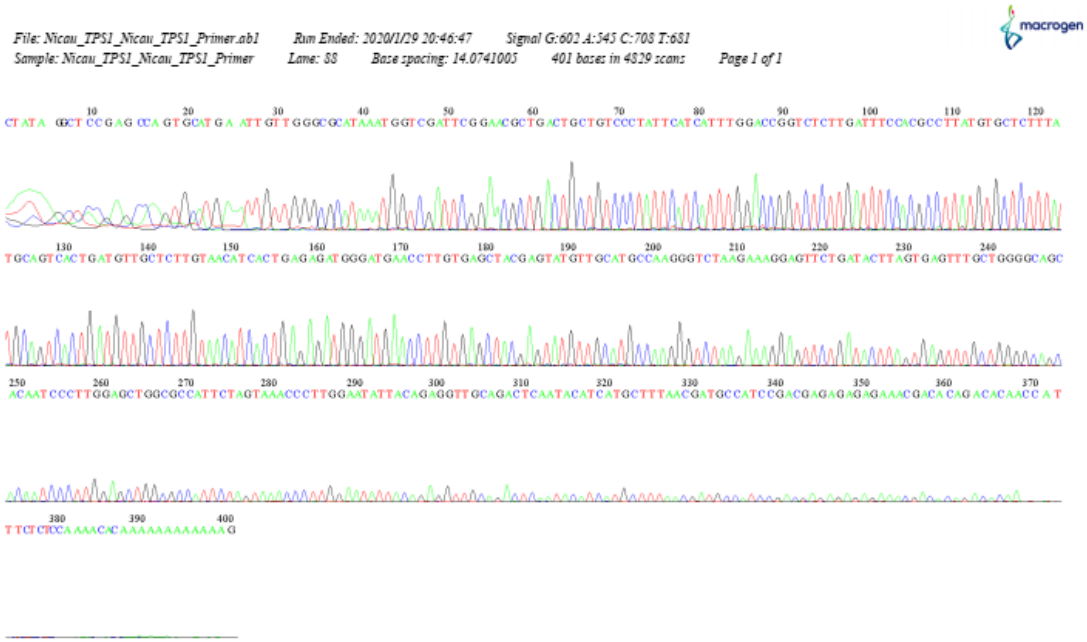
>Nicolau_*SoTPS1* primer



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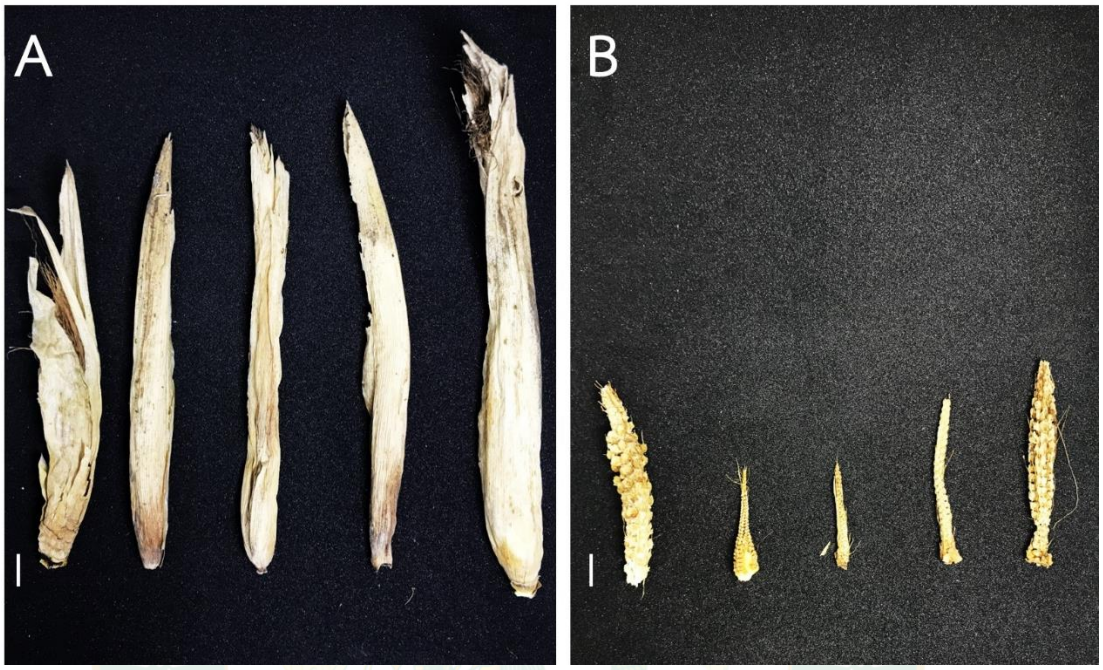
APPENDIX C

APPENDIX C

Maize ears of S_2 families under water stress condition for 7 days in greenhouse.



Phenotype of ear from S_2 maize families of some genotypes under water stress. For **A** is ear with husk and **B** is ear without husk. The white color score bar = 1 cm.

CURRICULUM VITAE

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