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Research article

Dot Blot Assay for Assessing Trehalose-6-phosphate Synthase Gene Expression in a Maize Breeding Population under Water Stress

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Abstract

Field maize is an important economic crop grown around the world and it has been mainly 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 for farmers. 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 (relative intensity at 64 DAP). However, dot blotting at 6 days after stress (relative intensity at 66 DAP) was effective to differentiate maize families. Furthermore, a moderate negative relationship between relative signal intensity at 66 DAP (RI_{66}) and Smith index based on multi-phenotypic traits was found to be statistically significant. 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.

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1. Introduction

Field maize (*Zea mays* L.) is an important economic crop in Thailand, and it is used as a raw material in the animal feed industry. The demand for field maize has been increasing not only in Thailand but also in foreign countries. Irregular rainfall during the growing seasons has shown to be a cause of drought problems in maize [1]. Under drought conditions, morphological and physiological traits of plants are often changed, e.g., shorter plant height, lower leaf area, earlier leaf senescence [2], shorter root length [3], longer anthesis-silking interval (ASI) [4]. Higher accumulation of compatible solutes such as trehalose and proline in plant cells is one of the mechanisms that plants use to protect themselves from water loss via osmotic adjustment [5, 6]. Trehalose acts as an osmo-protectant to protect cell membrane structures [7, 8] and is normally found at low [9, 7, 10, 11] or even undetectable [12] levels in plant cells under non-stress situations. However, the content of trehalose can increase substantially when plants experience abiotic stresses such as drought and salinity. The relationship between the levels of enzymes involved in trehalose biosynthesis and trehalose has been reported [13, 14]. Figure 1 shows trehalose biosynthetic pathway in plant cells. Trehalose phosphate synthase (TPS) and trehalose phosphate phosphatase (TPP) are two main enzymes in the trehalose synthesis pathway. Trehalose-6-phosphate (T6P) is an intermediate molecule that is synthesized and dephosphorylated by TPS and TPP enzymes, respectively (Table 1). Overexpression of *TPS* gene can increase the amount of T6P molecules in seedlings of *Arabidopsis thaliana* and *Oryza sativa* resulting in the higher content of trehalose detected [15-18]. Interestingly, these plants with overexpressed *TPS* gene showed more tolerant to water stress in those studies. A similar result was also found in sugarcane plantlets (56 days old) [19]. Therefore, it seems that higher accumulation of trehalose during stress has beneficial effect for plants. However, the estimation of trehalose content may be unreliable because it is often found at trace level [20, 21]. A molecular approach could be helpful in this case. To date, various types of molecular techniques have been used to estimate levels of gene expression including microarray analysis, reverse transcription-polymerase chain reaction, hybridization, and so on.

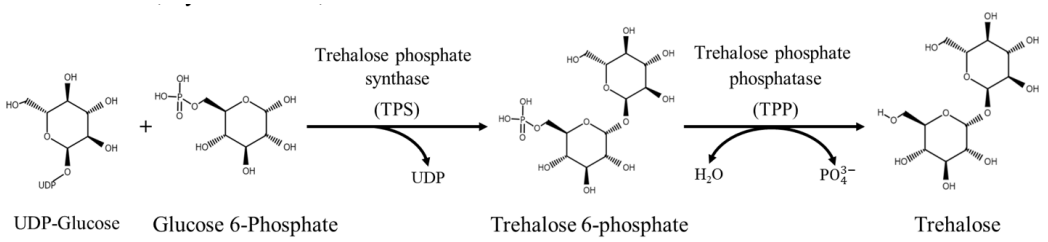

Figure 1. Trehalose biosynthetic pathway

Hybridization methods have been accepted as standard techniques for detecting particular sequences of either DNA or RNA, regardless 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) can hybridize with single strand mRNA of interest under optimal conditions. Beside the probe's specificity, this technique is simple, fast and has a low cost on sample preparation. It is used primarily for semi-quantitative analysis. Furthermore, large numbers of samples can be detected simultaneously.

As described, estimating gene expression involved in trehalose biosynthesis can be useful for maize breeding programs in order to gain more information about maize capability for osmotic adjustment and it might be used as an indicator for selecting drought tolerance. Therefore, the aim of this study was to screen the levels of *TPS* gene expression in maize breeding materials under water stress via dot-blot hybridization.

2. Materials and Methods

2.1 Developing cDNA probes

DNA sequence of *TPS* gene on Chromosome 8 of B73 maize reference genome (B73 RefGen_v4) (NM_001130121.2) was searched on NCBI nucleotide database and used for designing pairs of primer using primer-BLAST. Moreover, few pairs of published primers for *TPS* gene in rice [17] and sugarcane [19] that perfectly matched the same maize gene were used as well. Table 1 presents a list of primers that were used in this study.

To ensure a specificity of primers to *TPS* gene, the obtained PCR products were sequenced and checked for their similarity. To do so, total RNA was extracted from the leaves of tolerant maize seedlings (0.1 g) using TRIzol™ reagent (Thermo Fisher Scientific, USA). Total RNA was dissolved in 20 µl DNase-RNase free water and stored at -20°C for further use. Reverse transcription reaction was carried out to produce single strand cDNA using a Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) in a final volume of 20 µl. The RT reaction consisted of 2 µl of total RNA, 2 µl of 10 pmol specific primer, 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. For PCR, it was performed in 25 µl reaction containing 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 and subjected to a thermal cycler (Biometra Tone 96 G, Analytik Jena, Germany) with PCR profile as follows: 94°C for 5 min, 30 cycles at 94°C for 40 s, 50-58°C for 30 s (Table 1), 72°C for 2 min, and 72°C for 5 min. Subsequently, the PCR products were separated in 1% agarose gel electrophoresis at 100V for 40 min and visualized under UV-transilluminator. Purified PCR fragments for each pair of primers were obtained using a PCR Clean-Up and Gel Extraction Kit (Bio-Helix) before sequencing.

In order to label the cDNA probes, purified PCR products, which had been obtained from each pair of primer shown in Table 1, with concentrations of 500 ng-1 µg, were labeled using a DIG-High Prime DNA Labeling and Detection Starter Kit I (Cat. No. 11745832910, Roche, Germany). The standard protocol of the manufacturer (Cat. No. 11745832910, Roche, Germany) was followed. To check their efficiency, a serial dilution of DIG-labeled DNA standard (linearized DNA provided in the commercial kit) and all 4 labeled cDNA probes were spotted on a piece of nylon membrane to check their efficiency. According to the manufacturer's protocol, a series of 1 in 10 dilutions of DIG-labeled standard DNA and cDNA probes were recommended (1000, 100, 10 and 1 pg/µl). However, dilutions of 50, 30, 3 and 0.3 pg/µl were made and added in order to increase the resolution. Therefore, serial dilutions of 1000, 100, 50, 30, 10, 3, 0.3 and 0 pg/ul were prepared.

Table 1. Specific primer sequences for trehalose phosphate synthase gene and their characteristics

Accession number	Primer name	Sequence (5'-3')	%GC	Annealing temp. (°C)	Amplicon size (bp)	Ref.
NM_001130121.2	PH_*ZmTPS1-1* F	TACCAGGACGGGGATGTGAT	55	50	370	Newly-designed
	PH_*ZmTPS1-1* R	GCCTTTTCACTGCTGGAAGC	55			
NM_001130121.2	PH_*ZmTPS1-2* F	ATGGATTGGGTTGACAGCGT	50	58	550	
	PH_*ZmTPS1-2* R	TCGTGCTGCTGTGACTTGAT	50			
HM050424.1	Hao_*OsTPS1* F	TTGAAGTTCGGTCTGTTG	52	58	546	[17]
	Hao_*OsTPS1* R	CTGCCTATCCAAGAACATG	47			
EU761244.1	Nic_*SoTPS1* F	GTGCCAACAAGAACTGACG	44	55	400	[19]
	Nic_*SoTPS1* R	TGTGTCTGTGTCGTTTCTC	47			

Before spotting the DIG-labeled standard DNA and cDNA probes (Hao_*OsTPS1*, Nicolau_*SoTPS1*, PH_*ZmTPS1-1* and PH_*ZmTPS1-2*) at each dilution on membrane, a piece of nylon membrane was soaked with 10X SSC and then air-dried before performing the dot blot assay. After that, the spotted membrane was fixed under UV-light and washed with 1X maleic acid buffer (0.1M maleic acid, 0.15M NaCl, pH 7.5) at room temperature and followed a manufacturer's standard protocol for a color signal detection.

2.2 Preparing and testing plant materials

Thirty-four S_2 maize families, which had been coded as A1 to A34 and developed from the previous project [22], were grown in the greenhouse. Giving more detail about these S_2 maize families, these maize families had been developed from open-pollinated Suwan-1 field maize that had been hybridized with a few unknown landraces by local farmers. To exploit the events of allele recombination over time, these seeds with broad genetic background had been self-pollinated and develop into S_2 families to increase variance of additive gene effect [22].

This experiment was arranged in a completely randomized design with 3 replications. Water management, leaf samples and data collection on each day after planting (DAP) are shown in Figure 2. Different shades of colors are displayed on the bars of DAPs (Figure 2). Green represents a well-watered situation and healthiness of maize plants (before water stress) phase whereas orange shows a 'during stress' phase that was 7 day long (61-67 DAPs). Lastly, blue represents an 'after stress' phase. For water stress induction, water stress was placed on S_2 maize families for 7 days during the flowering stage. To do so, water was withheld for 7 days in advance (at 53 DAP) until the low level of soil moisture was read at 60 DAP (flowering stage), and this was considered as a 'during stress' phase (orange shade). After the 'during stress' phase, watering was resumed at 68 DAP (blue shade)

Leaf samples in the phases 1) 'before stress' (44 and 50 DAPs), 2) 'during stress' (62, 64 and 66 DAPs), and 3) 'after water stress' (69 DAP), were collected for dot blot assay. In addition, phenotypic traits, e.g., leaf greenness (SPAD) and leaf rolling (LR) were also measured at three DAPs (50, 62 and 64 DAPs) as shown in Figure 2. A symbol of SPAD and LR with subscription,

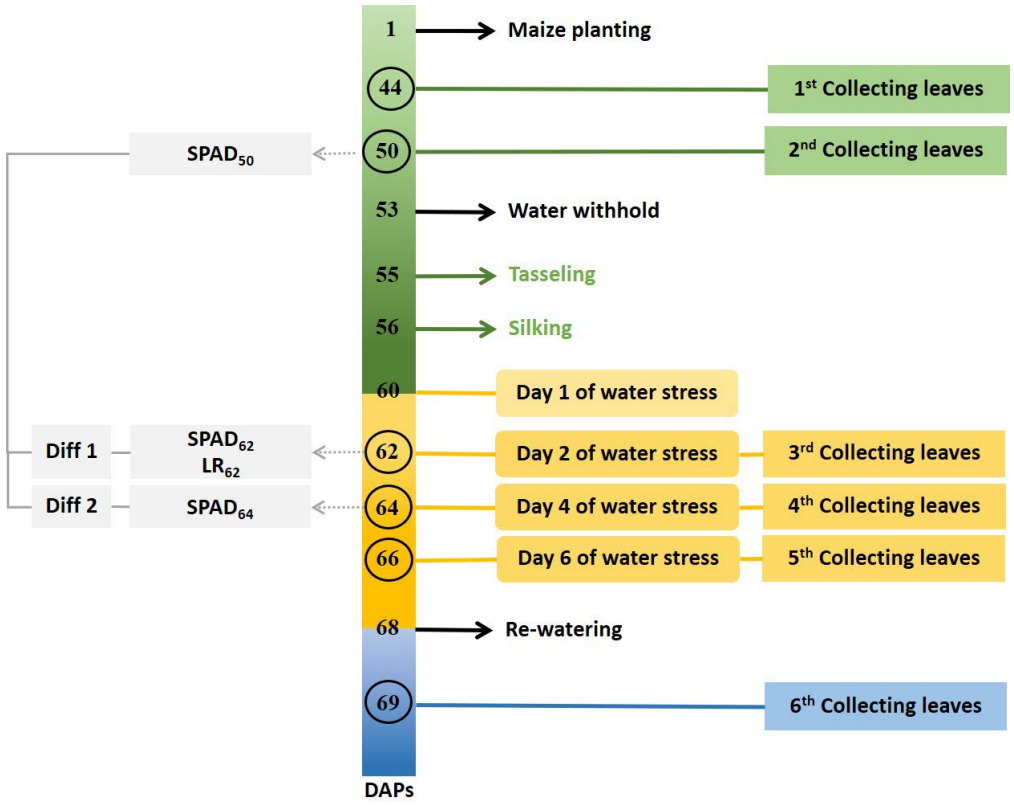

Figure 2. Experimental management at each day after planting (DAP)

i.e., $SPAD_{50}$, LR_{62}, etc., indicated the name of the trait at a specific DAP. SPAD units were measured using SPAD-502 plus chlorophyll meter (Konica Minolta) whereas LR in this study was the quantitative measurement of rolling leaves, which was measured in unit of centimeters. Plants with high LR were considered as being more tolerant since they could maintain themselves against water stress better than plants with lower LR. Furthermore, a change of SPAD units while maize plants were 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 indicate which maize families were able to maintain normal morpho-physiological traits longer over periods of stress.

2.3 Screening plant materials via dot-blot hybridization with imaging analysis

Maize leaves collected from each 6 different DAPs (Figure 2) were homogenized in 300 µl extraction buffer (50 mM sodium citrate, pH 8.3). Crude extracts were centrifuged at 7,000x g for 5 min, at 4°C. Then 3 µl supernatants were spotted onto 10X SSC-soaked nylon membranes and the standard protocol of the DIG-High Prime DNA Labeling and Detection Starter Kit I (Cat. No. 11745832910, Roche, Germany) was followed. In order to compare levels of gene expression among the maize samples, an estimation of the degree of relative signal intensity for *TPS* gene expression of all maize families were performed by scanning on the nylon membrane with a Scanner (Canon LiDE 400, Japan). Each image file was processed with ImageJ [23] and further analysis was carried out [24].

2.4 Statistical analysis

To estimate maize performance based on multi-phenotypically responsive traits (Diff1, Diff2 and LR_{62}), Smith selection index can be used as a predictor for this purpose [25]. With the concept of unequal importance of traits for selection, Smith selection index (*I*) includes weight for each trait as seen in the following [25, 26]:

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

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. In this study, Smith selection index was obtained via RindSel software [27]. Diff1, Diff2 and LR_{62} were subjected to obtain Smith selection indices for all 34 S_2 maize families. To find the best and worst families based on Smith index, a distribution of Smith index was constructed via histogram plot (data not shown) and 10% of two tail distribution was determined as a cut-off. So, those families from both tails were considered as the most tolerant and susceptible to water stress.

Moreover, one-way ANOVA analysis was performed to test the significant effect of maize family on 3 responsive traits and relative intensities at each DAP by using R statistical software [28]. Pearson correlation coefficients with a significance test at alpha 0.05 were also estimated for phenotypic traits, the relative signal intensity at all 6 different DAPs was estimated using STAR software [29]

3. Results and Discussion

3.1 Efficiency of DIG-labeled cDNA probes

All amplified fragments from 4 pairs of primers (Table 1) showed 91-99% similarity to *Zea mays* L. B73 reference genome, in which our 2 new designs of primers were 99% similar to *TPS*1 gene of maize reference genome. It indicated that Thai maize, from which leaf sample was initially used to be the template to synthesize PCR products with newly designed primers, had a variation with temperate B73 maize genome. However, this 99% similarity was acceptable. Moreover, Hao_*OsTPS1* and Nicolau_*SoTPS1* shared 94 and 91% similarities with the maize genome. Furthermore, A BLAST result showed that PH_*ZmTPS1-1* primer shared 89% similarity to *TPS* gene of *Oryza sativa* L. whereas the other 3 pairs of primers were more similar to *Saccharum officinarum* L. in the range of 94-96%. In addition, all 4 fragments also shared similarity with other species in family Poaceae, e.g., *Sorghum bicolor* L. (81-95%), *Panicum hallii* (89-94%) and *Setaria italica* L. (89-94%).

The efficiency of the 4 labeled cDNA probes is shown in Figure 3. As seen, signals from PH_*ZmTPS1-1* cDNA probe at 50 pg/µl dilution could be visualized compared with the other 3 probes at the same dilution, which were barely observed. It was likely that PH_*ZmTPS1-1* cDNA probe was the best here compared with the others (Figure 3). 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 image analysis.

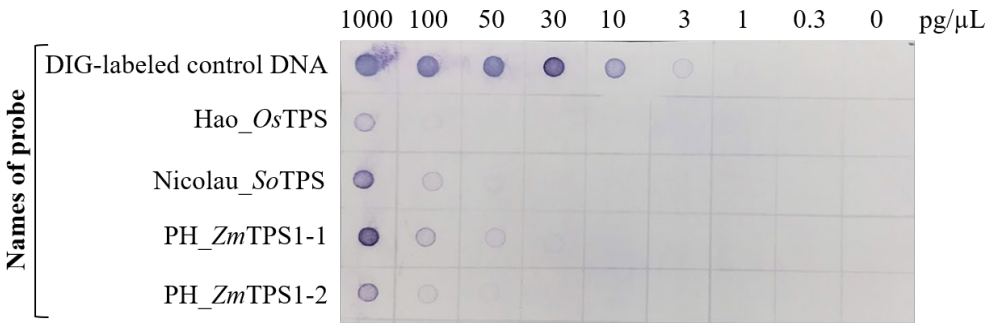

Figure 3. cDNA probe sensitivities across dilution series via Dot blot hybridization

3.2 Phenotypic analysis and Smith indice estimation for 34 S_2 families

Six phenotypic traits, e.g., $SPAD_{50}$, $SPAD_{62}$, $SPAD_{64}$, Diff1, Diff2 and LR_{62}, were collected at different DAPs, as shown in Figure 2. In general, combinations of plant growth stage, levels of soil moisture, and stress duration are the key factors to determine the levels of stress (mild, moderate or severe) of plants. Maize during flowering time is sensitive to stress and tends to lose up to 80% of yield [30-32]. Because water stress (7 day long) was given while maize plants were in their reproductive stage in this study, our maize experienced severe stress. Consequently, no harvested ears could be obtained. Therefore, the responsive traits, e.g., Diff1, Diff2 and LR_{62} were mostly used for the analyses. Diff1 and Diff2 could indicate the stay-green phenotype. In other word, delayed leaf senescence (stay-green) indicates the performance of maize to maintain normal metabolic processes under abiotic stress [33-38]. A study suggested that a lower rate of chlorophyll loss was often used as an indicator for selecting potato plants with drought tolerance [33]. Moreover, in this present study, the effect of maize family on Diff1, Diff2, LR_{62} and all 5 signal intensities was tested by performing one-way ANOVA. The results in Table 2 showed the strongly significant effect of maize family on 3 responsive traits only and not on any relative intensities. This revealed that the variability among 34 S_2 maize families existed and the data might be useful for our maize breeding program.

For estimating Smith index for all 34 families, Diff1, Diff2 and LR_{62} were used as previously described. According to Table 2, it was noticed that the lower values of Diff1 and Diff2, the better performance of those families based on Smith index. From Table 2, the Smith indices ranged from (-16.264) to (-124.117). At 10% cut-off of two-tail distribution of Smith index, it was found that maize families with codes A28, A10, A16 and A6 (order 1-4) and A32, A31, A23 and A22 (order 31-34) were considered as relatively drought tolerant and susceptible families, respectively. Moreover, it was noticed that the relatively drought tolerant families had average Diff1 and Diff2 values much lower (3-4 times) than those of the relatively susceptible families. High values of Diff1 and Diff2 often reflected early leaf senescence phenotype, which is a sign of less tolerance. This result of ranking maize families based on their Smith index corresponded to our previous project [22]. Breifly, seedlings of A10, A16 and A6 under osmotic stress (Polyethylene Glycol-6000 solution was used) showed vigour compared with the other 5 families. The same pattern was observed for mature maize. Therefore, it is likely that A10, A16 and A6 should be tested and used for a future project.

Table 2. Means of 3 phenotypic values and 5 relative signal intensities of chosen maize families based on 10% cut-off at two-tail distribution of Smith index from all 34 S_2 maize families

Order	Code of maize family	Origin	Smith index	Diff1	Diff2	LR_{62}	Relative signal intensity at each DAP				
							Before stress		During stress		
							44	50	62	64	66
1	A28	Grp3-13-1S1	-16.26	-3.91	6.19	9.70	0	0.23	0.15	0.00	0
2	A10	Grp0-11-2S1-4	-26.54	2.45	8.26	6.74	0	0	0	0.22	0
3	A16	Grp4-4-2S1-3	-30.12	2.66	7.78	9.00	0.19	0	0.43	0	0
4	A6	Grp0-4-S1	-31.17	1.9	10.81	8.23	0	0	0.0	0.11	0
	mean		-26.02	0.78	8.26	8.2	0.05	0.06	0.14	0.08	0
31	A32	Grp6-2-2S1-1	-81.92	14.93	26.38	11.36	0.19	0.00	0.56	0.44	0.25
32	A31	Grp6-2-1S1	-91.77	19.08	30.88	8.93	0.21	0.00	0.27	0.26	0.28
33	A23	Grp2-6-2S1-2	-101.00	18.38	39.16	9.01	0	0.22	0.29	0.23	0.21
34	A22	Grp2-6-2S1-1	-124.12	30.20	38.86	9.05	0	0.21	0.18	0.22	0.28
	mean		-99.70	20.65	33.82	9.59	0.10	0.11	0.33	0.29	0.25
		Overall mean	-49.05	6.17	16.72	9.39	0.08	0.06	0.28	0.21	0.11
		SD	23.22	6.42	8.76	1.50	0.11	0.10	0.17	0.17	0.13
		***p*-value**		1.99×10^{-7}	1.43×10^{-8}	3.36×10^{-4}	0.783	0.843	0.176	0.202	0.487

Note: Diff1 and Diff2 were different values between $SPAD_{50}$ with $SPAD_{62}$ and $SPAD_{66}$, respectively and LR_{62} was leaf rolling.

According to Table 2 and Figure 4, it seemed that the average relative signal intensities at all 5 DAPs from the top four families (green dots in Figure 4) were lower than those of the last four families (orange dots) even though no significant effect of family was found on all five signal intensities as described (Table 2). However, since Pearson correlation coefficients (r) between Smith index and these 5 relative intensities were calculated (data not shown), it was found that only a pair of Smith index with relative intensity at 66 DAP showed statistical significance at alpha 0.05. İts correlation coefficient was equal to -0.42 (p-value = 0.0128). This moderate negative relationship of Smith index with relative intensity at 66 DAP revealed that for better performance of maize families, lower TPS gene expression at 66 DAP was detected.

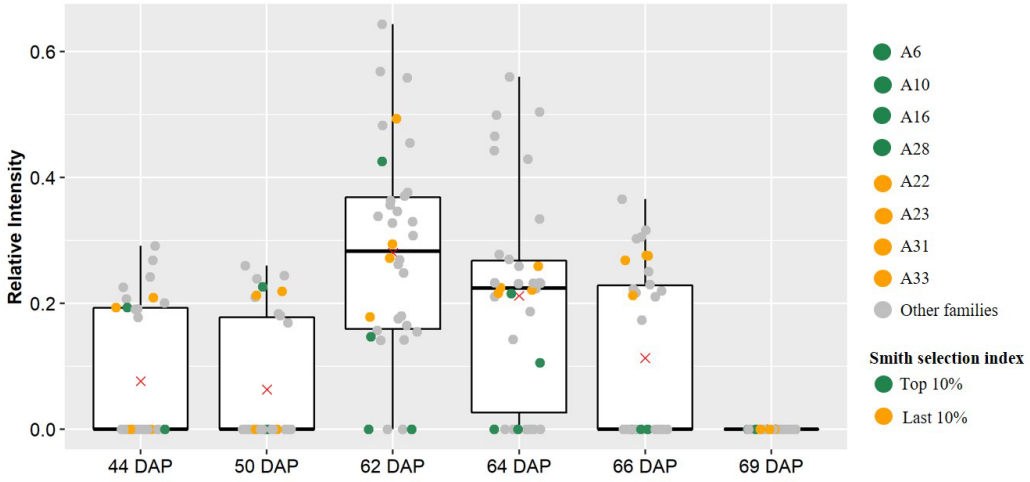

Figure 4. The level of relative intensity of dot blot signals of 34 S_2 maize families across 6 different DAPs. A red cross mark at each DAP indicates the overall mean for all maize families.

3.3 Screening breeding materials from 6 different days after planting via dot blot assay

Levels of relative signal intensity (RI) for all 34 maize families across 6 DAPs, e.g., 44 and 55 ('before stress' period), 62, 64 and 66 ('during stress' period) and 69 ('after stress' period) are illustrated in Figure 4 and some of them are presented in Table 2 as described earlier. According to Figure 4, the red cross marks represent the overall mean of RI of *TPS* gene expression for each DAP. It was interesting to observe a change of overall mean of RI across time periods (44 to 69 DAPs). The peak of average RI across DAPs was at 66 DAP, after which it decreased and became undetectable at 69 DAP (one day after re-watering). Many studies reported the same pattern [39-42].

Although ANOVA results (Table 2) showed non-significant difference of RI at all 5 DAPs among 34 maize families (p>0.05), it was clear that data points of RI at 66 DAP for 34 maize families fell separately into 2 tiers (top and bottom of box plot) whereas no specific pattern was found for both 62 and 64 DAP. This suggested that the detection of *TPS* gene expression at longer period of stress (66 DAP) could be used to classify maize families.

According to our results, the detection of *TPS* gene expression in mature maize via dot blot assay might provide a useful way to seek some potential maize families from the bulk. However, our results pointed out that higher levels of *TPS* gene expression under prolonged stress seemed to

be an unfavorable trait and it was contrast to other studies on *TPS* gene in seedlings or young plants [15-18, 12]. Some studies reported that plants with overexpression of *TPS* gene were likely tolerate to drought conditions. Overexpression of *TPS* gene in rice seedlings caused the higher accumulation of trehalose in the shoots, which was a 3- to 9-fold increase over the wild type [13]. This was similar to that found for young plantlets of tolerant and susceptible sugarcane, which was found that 56-day-old tolerant sugarcane had higher trehalose content than the susceptible one under water stress [19]. Nonetheless, it must to be noted that our results were obtained using mature maize that had experienced water stress during their flowering time.

3.4 Relationship between phenotypic traits and relative signal intensity

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 are presented in Table 3. Pearson correlation coefficients were in the range of -0.9 to 0.71. According to Table 3, 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 over a longer period of stress duration might be helpful for selection of the stay-green phenotype, which is one of the desirable traits for drought tolerance in maize. However, a lower level of TPS expression during prolonged period of drought stress is favorable to be selected for. In contrast to the stay-green trait, leaf senescence is caused by chlorophyll degradation which many plant species go through during drought stress [8, 43]. This eventually causes early leaf senescence and barren plants. *TPS* gene is upregulated when plants experience abiotic stresses [44, 45]. Furthermore, the levels of T6P (intermediate molecule in trehalose biosynthetic pathway) in mature plants were reported to be higher in early senescing leaves [46].

4. 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. 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 and A16 based on their Smith indices might have some potentials for further use in our breeding program.

Table 3. Pearson correlation between phenotypic traits and relative intensities of *TPS* gene expression

Variables	RI_{44}	RI_{50}	RI_{62}	RI_{64}	RI_{66}	$SPAD_{50}$	$SPAD_{62}$	$SPAD_{66}$	Diff1	Diff2
RI_{50}	0.004	1								
RI_{62}	-0.08	**-0.2**	1							
RI_{64}	-0.01	0.06	-0.19	1						
RI_{66}	-0.02	-0.03	0.17	-0.15	1					
$SPAD_{50}$	-0.11	-0.04	0.11	0.06	-0.02	1				
$SPAD_{62}$	-0.03	-0.07	-0.08	0.03	**-0.28**	0.18	1			
$SPAD_{66}$	-0.17	-0.11	-0.07	-0.03	**-0.21**	0.08	**0.66**	1		
Diff1	-0.04	0.04	0.14	0.01	**0.25**	**0.42**	**-0.82**	**-0.56**	1	
Diff2	0.1	0.08	0.11	0.06	0.19	**0.37**	**-0.54**	**-0.9**	**0.71**	1
LR_{62}	0.07	-0.14	0.06	0.14	0.03	0.07	-0.01	-0.1	0.05	0.12

Note: RI = Relative intensity of *TPS* gene at each day of planting, SPAD = leaf greenness, Diff1 and Diff2 = differences value between $SPAD_{50}$ with $SPAD_{62}$ and $SPAD_{66}$, respectively and LR = leaf rolling. The bold text values show statistically significant differences ($p<0.05$)

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