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Effect of thermal stress on amino acid and gene expression profiles in two local flax varieties with contrasting cold tolerance

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ABSTRACT

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Environmental stresses such as cold and heat are adversely affecting all aspects of crop plants including yield. In this study, the contents of fourteen amino acids and expression levels of four transcription factor genes including *MYB1-1*, *KRP2*, *ERF* and *WRKY40* were analyzed in TN-97-2 (cold sensitive) and TN-97-290 (cold tolerant) local flax varieties under cold and heat stresses conditions. Seeds of two local flax varieties were grown in growth chamber of Biotechnology Institute of Urmia University, Urmia, Iran, under control conditions for 30 days. Then, plants were subjected to three different thermal regimes including control (25± 1°C), cold (4 ± 1°C) and heat (37 ± 1 °C) for three consecutive days. Cold stress significantly increased the Asp, His, Ala and Met amino acids contents in both varieties. TN-97-290 variety exhibited less decline in His, Val, Phe, Iso and Leu contents than TN- 97- 2 variety under heat stress. Cold stress increased expression of *ERF* and *WRKY40* mRNAs while heat stress elevated transcript levels of *KRP2* and *MYB1-1* genes in TN-97-290 variety. In summary, Asp, Glu, His, Ala and Met amino acids could be exogenously applied to flax plants by either foliar spray or root supplement to tolerate cold stress conditions. In addition, application of Ser and Pro amino acids may aid conferring heat tolerance to cold sensitive flax plants. The positive response of *ERF* and *WRKY40* genes (cold stress) and *KRP2* and *MYB1-1* (heat stress) in TN-97-290 variety suggests their over-expression assist protecting flax plants under cold and heat stresses.

Keywords: flax, cold stress, heat stress, thermal tolerance, transcription factor

INTRODUCTION

Flax (*Linum usitatissimum* L.) belongs to Linaceae family and is originated from Mediterranean regions and/or south-west Asia (Millam *et al*., 2005). The plant is one of the earliest cultivated and important crops for the production of fiber, oil, and nutraceuticals (Vaisey *et al*., 2003). The cultivation of flax in Iran was practiced since 5000 BC and is one of the ancient crops in this country. About 15 flax species grow in Iran (Ghanavati, 2016). Nowadays, the crop has gotten considerable attention due to its importance.

Cold stress, classified as freezing (<0 °C) or chilling (0-15 °C, decreases membrane fluidity and induces membrane damage, water loss and oxidation with subsequent enhancement of reactive oxygen species (ROS) generation (Taibi *et al*., 2018). Heat stress, defined as temperatures > 10 °C above normal growing temperatures, impacts vegetative, flowering and seed filling stages resulting a reduction in seed set (Anon., 1996) and seedling growth (Hong and Vierling, 2001). Heat stress is a hot topic in the world bringing great challenges by adversely affecting crop plants and their yield (Katiyar-Agarwal *et al*., 2003).

Plants accumulate amino acids as the most common response to abiotic stresses by rising up the concentration to the millimolar range

(Singh *et al*., 1972; Planchet *et al*., 2011). Amino acids contain nitrogen, carbon, hydrogen, oxygen, with an organic side-chain in their structures (Zhao *et al*. 2016). They play their multifarious functional roles in abiotic stress tolerance via three mechanisms: 1- acting as compatible osmolytes, 2- regulating pH, 3- acting as a nitrogen or carbon reserve (Ali *et al*., 2019). Accumulation of amino acids improve stress tolerance in plants through regulation of gene expression, synthesis and activity of enzymes, detoxification of ROS, osmotic adjustment (Silvente *et al*., 2012; Zhao *et al*., 2016), regulation of ion transport and modulation of stomatal conductance (Zhao *et al*., 2016). Evidences indicating amino acids are accumulated at higher levels in stress-tolerant plants than sensitive plants support their protecting roles. Moreover, correlation between stress tolerance and amino acid levels has been demonstrated (Ali *et al*., 2019).

Plants also cope with adverse temperature conditions via regulation of stress related transcription factors (Zhou *et al*., 2008). The superfamily of MYB transcription factors comprised of the most diverse members in plants with involvement in plant development, secondary metabolism, hormone signal transduction, disease resistance and abiotic stress tolerance (Katiyar *et al*., 2012). A class of *CDK-inhibitory proteins*, the *Kip-related proteins* (*KRPs*), are negatively involved in *cyclin dependent kinases CDKs* (De Veylder *et al*., 2001). They regulate cell cycle checkpoints in response to oxidative STRESS (Yi *et al*., 2014). The *ERF* (*Ethylene Response Factor*), one of the largest subfamilies of *Apetala 2 (AP2)/ERF* transcription factors are also one of the important key regulators with diverse functions including stress regulation through the modulation of several signaling pathways (Rehman and Mahmood, 2015). *WRKY* gene family are one of the largest gene families of transcription factors in plants with wide range of biological funtions including response to biotic and abiotic stresses (Bakshi and Oelmüller, 2014; Chen *et al*., 2012).

Understanding of changes in amino acids contents and expression of transcription factors in plants with contrasting cold tolerance will help to identify the key metabolic processes controlling thermal tolerance. This study was conducted to reveal differential responses of two local flax varieties to cold and heat stresses by assessing variation in essential and non-essential amino acids contents and expression of *MYB1-1*, *KRP2*, *ERF* and *WRKY40* transcription factors.

MATERIALS AND METHODS

Plant material, growth conditions and stress treatments

Seeds of two local flax varieties; TN-97-2 and TN-97-290, were obtained from Agriculture and Natural Resources Research and Education Center of Urmia, West Azerbaijan province, Iran (Table 1). These flax varieties were identified as cold sensitive and tolerant, respectively as reported earlier (Ghoreishi *et al*., 2017). Seeds of two local flax varieties were surface sterilized using 75 % ethanol (v/v) and germinated on filter paper moistened with distilled water at 8/11°C in the dark for five days. Uniformly germinated seeds were transplanted in pots (20 cm diameter) filled with a transplanting medium containing peat moss, vermiculite and perlite (10:1:1). Each pot contained four plants and was considered as one replication. The experiment was carried-out in three replications. The plants were grown in growth chamber for 30 days at 25 ± 1°C with 16 hours day light and 8 hours darkness at 100 µmol m^{-2} s^{-1} photon flux density using cool-white fluorescent light. Thirty-day old flax plants were subjected simultaneously to three different temperature regimes including control (25 ± 1°C), cold (4 ± 1°C) and heat (37 ± 1 °C). After three days, plants were harvested and immediately placed in liquid nitrogen and stored at -80 °C (Savitch *et al*., 2001).

Table 1. Some agronomic and quality characteristics of two local flax varieties

Variety	Number of branches	Plant height (cm)	1000 seed Weight (g)	Capsule weight plant^{-1}(g)	Number of capsules Plant^{-1}	Oil Yield (kg ha^{-1})	Seed yield (kg ha^{-1})	Biological yield (kg ha^{-1})	Oil content (%)	Protein content (%)	Protein Yield (kg ha^{-1})
TN-97-2	6.45	20.7	4.52	2.22	16.9	352.15	992	1993.9	36.3	24.06	351.3
TN-97-290	7.35	27.02	5.0	3.92	12.72	459.83	1461.47	2986.7	31.78	24.14	397.09

Amino acid analysis

The content of fourteen amino acids including eight essential amino acids: Methionine (Met), Leucine (Leu), Lysine (Lys), Phenylalanine (Phe), Tyrosine (Tyr), Arginine (Arg), Isoleucine (Iso) and Valine (Val) and six non-essential amino acids: Aspartic acid (Asp), Glutamic acid (Glu), Serine (Ser), Proline (Pro), Histidine (His) and Alanine (Ala) were determined. All leaf samples were analyzed using Shimadzu HPLC interfaced with SPD-10 AVP series, variable wavelength (190-750nm), UV-Vis detector (Kurilich *et al*., 1999). The extracts were centrifuged at 12,000g for five minutes by variable speed refrigerated multiple rotor centrifuge K280R, United Kingdom. To 50 µl of supernatant, 50 µl ortho-phthaldialdehyde amino acid (OPA) was added, and the reaction mixture was derivatized by adding 20 µl of 9-fluorenylmethylchloroformate (FMOC). After derivatization, 10 µl norleucine was added as internal standard, thereafter the mixture was vortexed for two minutes, and 60 µl of extract was injected using Hamilton Syringe (model 1710 Small RN Valco VISF-1). After pre-column derivatization, amino acids were separated by reverse phase high-performance liquid chromatography (RPHPLC) (KNAUER Smart line HPLC-System Germany) using C-18 column. Calibration of the system was carried out using amino acid standards (Sigma) with the addition of 0.2 mM norleucine.

Total RNA extraction and cDNA synthesis

Total RNA was extracted according to the modified CTAB method (Gasic *et al*., 2004). To eliminate DNA contamination, total RNA was treated with DNase I (Ambion, USA), then purified according to the manufacturer's protocol. The integrity of total RNA samples was verified by 1.2% (w/v) agarose gel electrophoresis, and the quantity and quality of RNA samples were measured with the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA). To perform qRT-PCR, cDNA was synthesized by reverse transcription using 2.5µg total RNA in a 20 µl reaction volume according to the manufacturer's instructions (Thermo Scientific, USA). The cDNA was diluted 10-fold with nuclease free water for qRT-PCR analysis.

qRT-PCR analysis

qRT-PCR reactions were performed in 20 µl system on a Bio-Rad CFX96 Real-Time PCR system (Bio-Rad, USA) with a reaction contained 10 µl of Fast Start Universal SYBR Green Master (Roche, Germany), 2 µl of diluted cDNA template, 1µl of each primer (10 µM). For each gene, a no template control (NTC) was included using water instead of cDNA as template. The qRT-PCR reactions were conducted following the fast thermal cycles: 95 °C for 10 minutes, 40 cycles at 95 °C for 30 seconds, 58 °C for 30 seconds, and 72 °C for 30 seconds. After 40 cycles, the dissociation curve was performed to confirm the specificity of each primer again by heating up the product from 60 °C to 95 °C. The Rn (normalized reporter) threshold was automatically selected to obtain the cycle threshold (Ct) values. The final Ct value of each sample was the mean of three biological replicates and three technical replicates. The mean amplification efficiency of each primer pair was checked by the Lin Reg PCR program (Ruijter *et al*., 2009). *EF1a* gene was used as an endogenous reference. The specificity of the primers was confirmed using the single peak melting curves of the qPCR products (Table 2).

Table 2. Primer sequences and description of genes

Gene	Primer sequence	Reference	Gene description
WRKY40	F: GGCAGAAGGTGACCAGAGAT R: CCACTACCGATTGGTCCTCC	(Huis R *et al*., 2010)	*DNA-binding protein 40*
EF1A	F: GCTGCCAACTTCACATCTCA R: GATCGCCTGTCAATCTTGGT	(Huis R *et al*., 2010)	*Elongation Factor 1-α*
ERF	F: GACCGCATTTGGTTGGGTTC R: GTGGGTCCACGTAAGCAGAA	(Huis R *et al*., 2010)	*Ethylene responsive factors*
MYB1-1	F: GAGGACATCCTCCTGGTCAA R: TCCCTCGTTTGAGATCCGGT	(Huis R *et al*., 2010)	*Myb domain protein 1-1*
KRP2	F: AGACCAACCTTCCCCAGATT R: CGGGAAGGGTCATCACGTT	(Huis R *et al*., 2010)	*Kip-related cyclin-dependent kinase inhibitor 2*

Statistical analysis

The experiment was conducted as factorial using completely randomized design with three replications. The relative expression level of the genes was calculated using $2^{-\Delta\Delta CT}$ value (Klein, 2002). To apply the $2^{-\Delta\Delta CT}$ method, the results of real time PCRs were represented as cycle threshold (Ct) values. Normality test of the data and residuals were performed using MINITAB16 software. Data were subjected to analysis of variance, and means were compared by Least Significant Difference (LSD) TEST.

RESULTS AND DISCUSSION

Amino acid analysis

The free amino acid pools differed between two varieties under different thermal conditions (Table 3 and Fig. 1). Cold stress (4°C) significantly increased the total amino acids to 48.88 µmolg^{-1} fr.wt in TN-97-2 variety while reduced it to 31.58 µmolg^{-1} fr.wt in TN-97-290 variety (Table 3). Under cold stress conditions, both varieties responded similarly to induce Asp, Glu, His, Ala and Met contents along with declines in Ser, Phe, Iso, Leu and Lys contents, compared to relevant control plants (Table 4 and Fig. 1). Under heat stress conditions, the total amino acid content was reduced to 32.42 µmol g^{-1} fr.wt and 29.15 µmol g^{-1} fr.wt in TN-97-2 and TN-97-290 varieties, respectively, compared to plants grown in control conditions (Table 3). This reduction appeared to be only significant for tolerant variety. Despite of the decline in concentration of most amino acids, heat stress significantly enhanced the Pro content in both varieties (Fig. 1). The TN-97-290 variety exhibited less decline in His, Ala, Val, Phe, Iso and Leu contents than TN- 97- 2 variety under heat stress conditions.

Table 3. Changes in the total amino acid content (µmol g^{-1} fr.wt) in two local flax varieties after three days thermal stress

	TN-97-2			**TN-97-290**		
	25˚C	**4˚C**	**37˚C**	**25˚C**	**4˚C**	**37˚C**
TEAA	12.53	12.75	8.22	11.56	9.97	7.92
TNEAA	20.33	36.13	24.19	21.77	21.6	21.235
TAA	**32.86^{c}**	**48.88^{a}**	**32.42^{c}**	**33.34^{b}**	**31.58^{d}**	**29.15^{e}**

TEAA: total essential amino acid; TNEAA: total nonessential amino acid, TAA: total amino acid

Table 4: Analysis of variance for amino acids of two local flax varieties grown under thermal stress

		Mean squares						
S. O. V.	d.f.	Asp	Glu	Ser	His	Pro	Arg	Ala
Temperature (T)	2	41.73**	90.28**	16.44**	0.415**	7.59**	2.20**	23.89**
Variety (V.)	1	4.39**	2.37**	0.038	0.11**	0.0.08**	0.86**	3.57**
T × V	2	1.15**	2.43**	5.30**	0.115**	5.08**	7.52**	1.24**
Error	10	0.13	0.0106	0.022	0.000	0.0007	0.033	0.044
CV (%)		14.88	5.66	2.68	1.22	0.133	3.61	4.33

Table 4. Continued.

		Mean squares						
S. O. V.	d.f.	Tyr	Met	Val	Phe	Ilue	Leu	Lys
Temperature (T)	2	3.87**	0.093**	0.86**	0.228**	0.344 **	0.200**	0.044**
Variety (V.)	1	8.59**	0.0007	0.21**	0.000	0.072**	0.032**	0.0004
T × V	2	3.26**	0.002**	0.22**	0.033**	0.055**	0.026**	0.0005
Error	10	0.05	0.0001	0.018	0.0015	0.0005	0.0023	0.0008
CV (%)		7.14	8.75	14.76	11.95	6.85	15.14	12.03

**: Significant at the 1% probability level.

Asp: Aspartic acid, Glu: Glutamic acid, Ser: Serine, His: Histidine, Pro: Proline, Arg: Arginine, Ala: Alanine), Tyr: Tyrosine, Met: Methionine, Val: Valine, Phe: Phenylalanine, Ileu: Isoleucine, Leu: Leucine, Ley: Lysine.

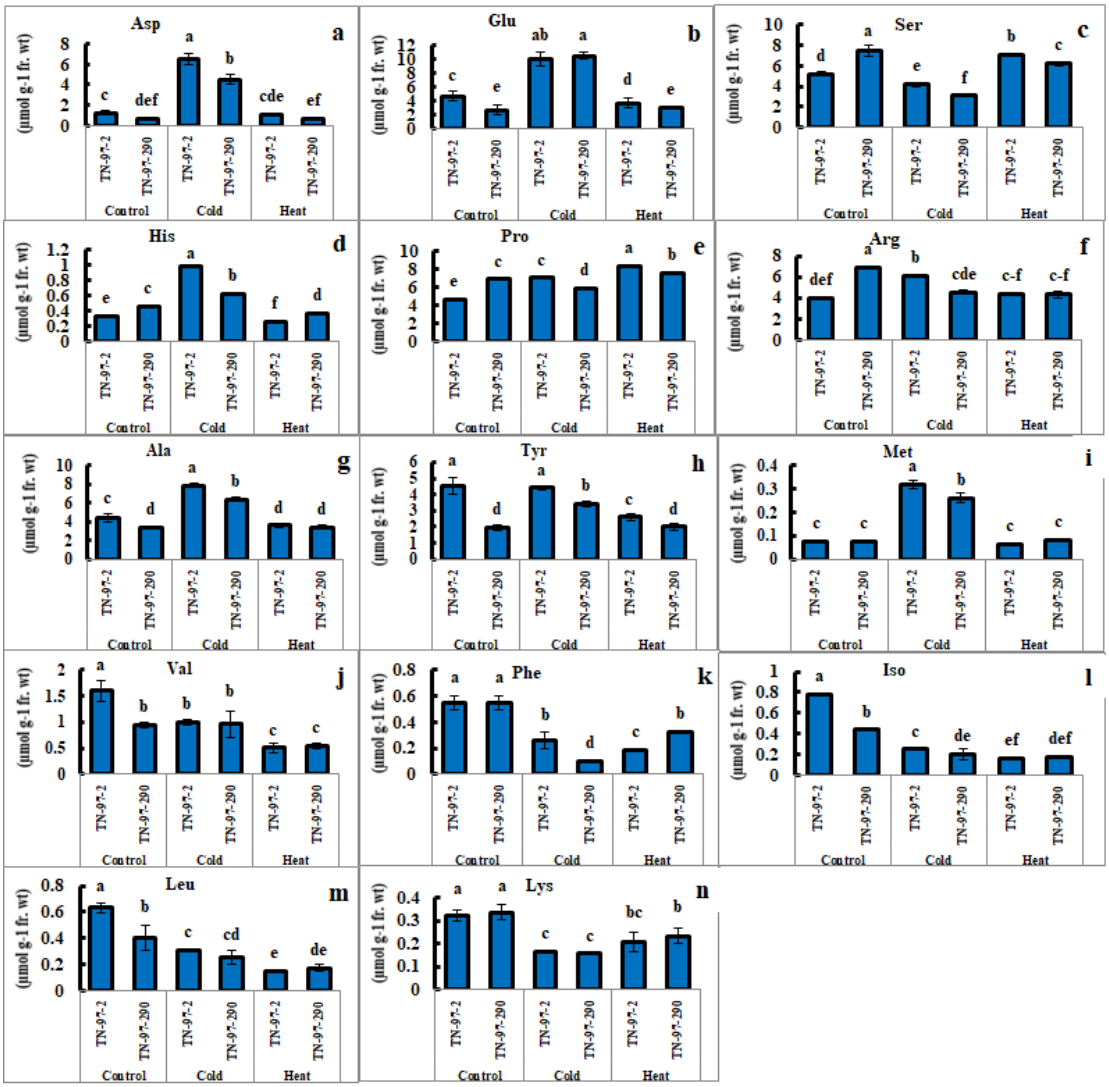

Fig. 1. Mean comparison of temperature × variety interaction effect on amino acids of two flax varieties grown under thermal stress. a) Asp (Aspartic acid), b) Glu (Glutamic acid), c) Ser (Serine), d) His (Histidine), e) Pro (Proline), f) Arg (-Arginine), g) Ala (Alanine), h) Tyr (Tyrosine), i) Met (Methionine), j) Val (Valine), k) Phe (Phenylalanine), l) Ileu (Isoleucine), m) Leu (Leucine), n) Ley (Lysine). Bars with similar letters are not significantly different at the 1% probability level-using least significant difference test

Several reviews have repeatedly reported the accumulation of free amino acids (Glu, Asp) in plants under abiotic stresses conditions (Yang *et al*., 2015; Hildebrandt 2018; You *et al*., 2019). Free amino acids accumulation may result from protein breakdown and/or synthesize under stress conditions by plants (Hildebrandt, 2018). They contain carbon, hydrogen, oxygen and nitrogen in their structure and are involved in production of proteins and growth substances (Zhao *et al*., 2016). They all play critical roles in plant growth, development and abiotic stresses tolerance. However, their effects are plant-type specific and amino acid-type specific (Ali *et al*., 2019).

Both local flax varieties expressed higher accumulation of Glu under cold stress exposure. Glu plays a central role in nitrogen metabolism and chlorophyll synthesis processes. The amino acid is a precursor of Pro, glutathione (GSH) and polyamines and interacts with abscisic acid signaling system which all protect plants against environmental adverse effects (Kovács *et al*., 2012; Yang *et al*., 2015; Wang *et al*., 2018). The

accumulation of Asp and Met induced by cold stress in both varieties suggested that they participated in resistant to cold stress in flax varieties. In accordance to our findings, induction in Asp and Met concentration in cold tolerant and cold sensitive *Pinus halepensisseed* plants has been previously reported under cold stress by Taibi *et al*. (2018). Another glutamate amino acid family, His, also increased in two local flax varieties under cold stress conditions which was consistent with previous reports in other plant species (Taibi *et al*., 2018).

Pro has been widely reported for accumulation in response to various abiotic stresses such as; drought, salt, and high and low temperatures (Shin *et al*., 2016). Based on our findings, cold tolerant variety (TN-97-290) exhibited slight decrease and mild increase under cold and heat stresses, respectively, indicating higher tolerance and more adaptatability of this variety in response to adverse thermal conditions (Fig. 1).

Tyr concentration increased in cold tolerant variety (TN-97-290) under cold stress conditions implying its role in enhancing cold tolerance. Try is aromatic amino acid and known as precursor for numerous secondary metabolites and its involvement in abiotic stress defense response, synthesis of phenolic compounds, alkaloids and cell wall components (Khan *et al*., 2019; You *et al*., 2019).

The level of branched-chain amino acids (BCAAs) (Leu, Iso, and Val) as well as Lys significantly decreased in both local flax varieties under heat stress conditions. However, this reduction was less pronounced in cold tolerant variety (TN-97-290) showing its higher tolerance to alteration. These findings were contrary to many previous studies that showed these amino acids increased under abiotic stresses conditions (Bowne *et al*., 2012; Wang *et al*., 2018; You *et al*., 2019). BCAAs play important roles in plant stress tolerance as an alternative source of respiratory substrates (You *et al*., 2019).

Expression profiles of TF genes

Analysis of variance of data showed significant effect of thermal treatments on genes expression (Table 5). Three days' exposure of flax plants to 4°C enhanced mRNA level of *MYB1-1* and *KRP2* gene in TN-97-2 variety by 1.8 fold, while reducing their mRNA levels in TN-97-290 variety compared to plants grown under control condition. Heat stress enhanced transcript level of *MYB1-1* by about 1.5 and 2.5 fold in TN-97-2 and TN-97-290 varieties, respectively, compared to control plants. The upregulation of *MYB1-1*and *KRP2* genes in TN-97-290 variety suggested that their over-expression in flax varieties may help in providing protection under heat stress conditions. Sheikh *et al*. (2008) reported higher *MYB1* expression under wounding, salt, drought and heat stresses in rice. Several members of *MYBs* are up-regulated by various abiotic stresses both in rice and Arabidopsis (Katiyar *et al*., 2012). The critical role of *KRP2* in growth inhibition has also been shown in model plants in response to drought and cold stresses (Sonju *et al*., 2005).

Table 5. Analysis of variance for for four studied genes in two local flax varieties grown under thermal stress.

S. O. V.	d.f.	*MYB1-1*	*KRP2*	*ERF*	*WRKY40*
Temperature (T)	2	2.67**	0.86**	0.71**	2.79**
Variety (V)	1	0.00**	0.26**	1.34**	4.53**
T × V	2	1.35**	3.22**	1.54**	5.26**
Error	10	0.06	0.21	0.04	0.26
CV (%)		20.67	40.29	26.26	51.68

**: Significant at the 1% probability level.

Exposure to 4°C raised *ERF* and *WRKY40* transcript levels in TN-97-290 variety (cold tolerant) under cold stress conditions, and heat stresses significantly reduced mRNA accumulation of *ERF* and *WRKY40* genes in both varieties compared to controls (Fig. 2). These observations indicated the contribution of *ERF* and *WRKY40* genes in adaptability of

flax varieties against low temperatures. Seventy four *ERF* genes *(AcoERFs*) were identified in pineapple genome, named from *AcoERF1* to *AcoERF74* (Huang *et al*., 2020). Several *ERF* genes such as *OsEREBP1*(Serra *et al*., 2013), *OsERF101* (Jin *et al*., 2018), *Sl-ERF.B.3* (Klay *et al*., 2014), *AhERF019* (Wan *et al*., 2014) and *OsEREBP1*(Jisha *et al*., 2015) were induced by salt, drought, low temperature and heat stresses as well as changes in light availability, respectively. Klay *et al*. (2018) revealed that a group of *ERFs* are preferentially associated with cold and heat stress responses. *WRKY* gene family, one of the largest families of transcription factors in plants, participates in diverse biological processes, including response to biotic and abiotic stresses (Bakshi and Oelmüller, 2014). Lee *et al*. (2005) have reported that eight *WRKY* transcription factors were all cold responsive and upregulated. Chen *et al*. (2008) have demonstrated that *WRKY40* is a transcriptional repressor in plant cells and together with *WRKY18* and *WRKY60* form a highly interactive regulatory network to modulate gene expression in both plant defense and stress responses.

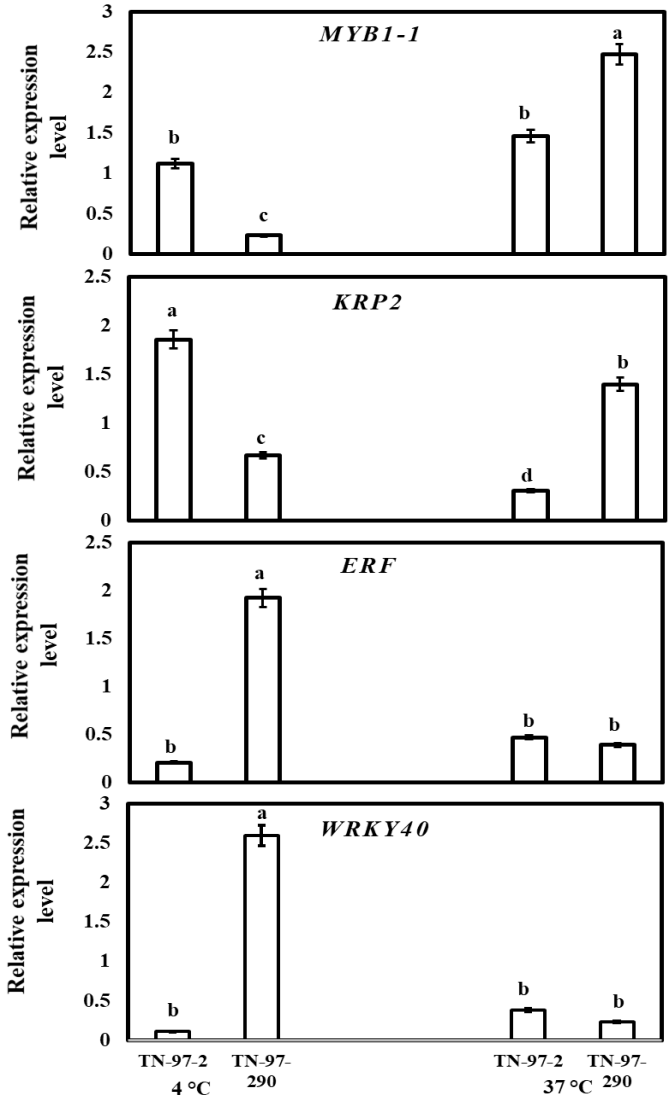

Fig. 2. Mean comparison for temperature × variety interaction effect on expression of *MYB1-1*, *KRP2*, *ERF* and *WRKY40* genes compared to control in two local flax varieties. Bars with similar letters are not significantly different at the 1% probability level-using least significant difference test

In conclusion, Asp, Glu, His, Ala and Met amino acids can be applied as foliar spray or root supplement to protect the two local flax varieties under unfavorable cold conditions. Our data also suggest that supply of Ser and Pro amino acids to cold sensitive local flax varieties can boost their survival under heat stress conditions. Based on gene expression analysis, the overexpression of *ERF* and *WRKY40* genes (under cold stress conditions) and *KRP2* and *MYB1-1* genes (under heat stress conditions) may also enhance protection of local flax varieties under conditions of thermal fluctuations.

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