RESEARCH ARTICLE Legume Research- An International Journal, Volume 44 Issue 1: 60-66 (January 2021)

Selection by Genetic Expression Profiles of *Desi* and *Kabuli* Chickpea (*Cicer arietinum* L.) Genotypes Tolerant to High Temperature Stress

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ABSTRACT

Background: Mexico is an important producer of chickpea; however, high temperatures during flowering and grain filling limit seed yield and seed size. Plant adaptation strategies to heat stress depend on climatic and soil conditions, but mainly on the plant genetic characteristics. The increase in heat shock proteins (HSP) production occurs when plants experience an abrupt or gradual increase in temperature in order to whithstand stress with the least damage.

Methods: Sixty-five Heat Shock Protein related genes that induce transcription under heat stress were studied according to their expression profiles. This strategy allows for the selection of chickpea genotypes bearing potential heat stress tolerance. Based on the number of overexpressed (induced) genes and on its level of expression, a tolerance index was calculated.

Result: Tolerant *desi* genotypes were: ICC 10259, ICC 13020, ICC 4958 and Annigeri; and in the *kabuli* type outstanding genotypes were: Mazocahui, ICCV 2, Blanco Sinaloa 92, Tequi Blanco 95, Combo 743 and CUGA 08-1210. These genotypes showed profiles with a higher number of induced genes and higher Tolerance Indexes. These genotypes will be further evaluated in the field and under controlled conditions and in the near future used as parental stocks.

Key words: Heat stress, HSP, Levels of expression.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the third most important grain legume worldwide. Developing countries are responsible for 96 % of its production, being India the leading producer; Mexico ranks in ninth place and produces two types of chickpea: white or *kabuli* and forage or *desi* type. The first one is produced in the northwest region for export purposes. The *desi* type is used mainly in the central-western region (Guanajuato, Jalisco and Michoacán) as livestock feed (Merga and Haji, 2019). High temperature is one of the main abiotic factors that reduces grain yield. Studies have found that temperatures above 30°C induce the plants to mature rapidly. Plant adaptation strategies to heat stress depend on climatic and soil conditions, but mainly on the plant genetic makeup. The increase in production of thermal shock protein (HSP) occurs when plants experience an abrupt or gradual increase in temperature (Devasirvatham and Tan, 2018). High temperatures produce protein unfolding that leads to the formation of protein aggregates and precipitates. Several HSPs function as chaperones and participate in the correct folding of other proteins and keep their conformation stable during the stress (Parankusam *et al*. 2010). Heat Stress Transcription Factors (HSFs) regulate the expression of HSPs by recognizing the heat stress elements (HSE, 5′GAAnnTTC-3′). There are five major classes of HSPs: HSP100, HSP90, HSP70, HSP60 and small heat shock proteins (sHSPs) (Iba, 2002; Guo *et al*. 2016). HSP101, HSP70, HSP17.7 and HSP17.6 are now known to protect plant cells from programmed cell death induced by stress at high temperatures (Rikhvanov *et al*. 2007). HSP21 protects the transcription of plastid-encoded RNA polymerase-dependent in chloroplast under heat stress (Zhong L. *et al*. 2013). Recently, Lin *et al*. (2018) described that the expression of miR160 is induced by heat stress and its activity regulates the expression of HSPs.

Biotechnological tools represent an opportunity to help reduce the losses generated by high temperatures throughout the identification of genes transcribed as a response to this stress, as well as the determination of their function (Gaur *et al*. 2018). In this research, we studied genes related to the heat stress tolerance and measured their expression levels by RT-PCR, results allowed us to identify *kabuli* and *desi* chickpea genotypes that potentially can tolerate high temperatures.

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How to cite this article: Rodríguez-Vera, A.P., Acosta-Gallegos, J.A., Ruiz-Nieto, J.E. and Montero-Tavera, V. (2021). Selection by Genetic Expression Profiles of *Desi* and *Kabuli* Chickpea (*Cicer arietinum* L.) Genotypes Tolerant to High Temperature Stress. Legume Research. 44(1): 60-66. DOI: 10.18805/LR-541.

Submitted: 12-12-2019 **Accepted:** 21-06-2020 **Online:** 28-09-2020

MATERIALS AND METHODS

Chickpea genotypes

Thirteen *kabuli* and sixteen *desi* genotypes were tested (Table 2). The *desi* type included ICC 4958, a drought-tolerant genotype and for which its complete genome has been sequenced (Parween *et al*. 2015). The ICC and ICCV materials come from the germplasm bank of ICRISAT and were included due to outstanding agronomic characteristics. The *kabuli* materials included fixed lines from the breeding Program of the National Research Institute for Forestry, Agricultural and Livestock (INIFAP).

Gene selection for expression studies

We searched the chickpea genome of genotype ICC 4958 (Jain *et al*. 2013) for sequences of HSPs. Sixty-five genes were identified for which we designed specific primers with the Primer-BLAST algorithm (Ye *et al*., 2012) and the company Sigma-Aldrich carried out their synthesis. With the aid of final point PCR we identified and used 35 sequences which produced only one amplicon (Table 1).

Treatments

Fifty seeds of each material were sown and ten morphologically uniform plantlets were selected and divided into two groups, five to be kept under non-stress conditions (26°C) and five under a high-temperature treatment during the vegetative stage, phase characterized by the appearance of secondary branches. When plants reached 32 days after sowing, the second group was kept for two hours in a MRC® growth chamber with the temperature set at 40°C.

Expression profiles

To determine the level in the expression of HSPs, a bulk of leaf tissue of each genotype under the tested conditions was used. From each bulk total RNA was extracted and purified following the protocol of Logemann *et al*. (1987), subsequently their concentration was standardized to 1,000 ng μL^{-1}. Single-stranded cDNAs were synthesized by reverse transcription using the Super SMART™ PCR cDNA Synthesis Kit, following the conditions suggested by the manufacturer. To determine the gene expression in response to both temperature treatments, the baseline level in samples from each treatment was normalized by amplifying the *26S* gene by RT-PCR according to the methodology of Montero-Tavera *et al*. (2017). The reactions, amplification program and documentation of results were carried out as Montero-Tavera *et al*. (2017).

Densitometric analysis of expression levels

The expression levels generated by RT-PCR were measured using the TotalLabQuant software. The relative expression of each gene was defined in relationship to the expression of the constitutive gene *26S* given it a value of 1. The selection criterion of induced genes was based on a completely random ANOVA analysis by calculating the minimum significant difference, which results of 0.1.

Identification of putative heat-tolerant genotypes

Based on the level of expression and in order to discriminate the genotypes, cluster analyses were carried out in both chickpea types. The relative expression values from all genes were used in a heatmap analysis with the heatmap.2 algorithm of the R statistical package gplots v3.0.1.1 (Warnes *et al*. 2015); distances were calculated with the Ward and Shakespeare (1998) coefficient. Furthermore, we calculated a modified Tolerance Index (TI) of Rosielle and Hamblin (1981) using as input the expression levels. This index represents the relationship of the expression under stress and non-stress conditions, which was normalized in each test considering the expression of the constitutive *26S* gene. TI was calculated as follows:

$$(TI) = \Sigma(a_1\text{-}b_1/c)/n$$

Where

a_1 = expression of gene 1 under normal temperature.
b_1 = expression of gene 1 under heat stress.
c = constitutive gene expression.
n = number of genes.

RESULTS AND DISCUSSION

RT-PCR expression profiles

Fig 1 shows the expression profiles of all genotypes exposed to both temperature treatments. The tolerant *desi* induced on average 30.5 genes, the moderately tolerant 23.8 and the susceptible 11.5; whereas in the *kabuli* type induced genes were 28.4, 20.7 and 10.5, respectively (Table 2). Ten genes were identified whose expression profile is useful for the selection of putative heat-tolerant genotypes in both types of chickpea (*CaHS3*, *CaHS5*, *CaHS19*, *CaHS22*, *CaHS26*, *CaHS37*, *CaHS38*, *CaHS41*, *CaHS56* and *CaHS58*); in addition to these, 8 exclusive genes were identified for the selection of *kabuli* genotypes (*CaHS1*, *CaHS10*, *CaHS12*, *CaHS15*, *CaHS21*, *CaHS24*, *CaHS46* and *CaHS53*) and 6 exclusive for the selection of *desi* genotypes (*CaHS14*, *CaHS16*, *CaHS43*, *CaHS48*, *CaHS59* and *CaHS63*).

Among the genes with a higher level of expression under stress in both types of chickpea, the ones that code for 70 kDa (HSP70) and 22 kDa (HSP22) proteins stand out. The expression of HSPs under normal conditions account for 5% of the total intracellular proteins; however, under stress conditions, they can increase up to 15% (Fragkostefanakis *et al.,* 2015). This agrees with our results and confirms that it is possible to determine expression profiles associated with heat stress response, which could be used in a selection program for the development of genotypes tolerant to this stress.

Desi genotypes induced a higher number of genes. We observed in all genotypes the induction of CaHS22 and CaHS37 genes, corresponding to a non-yet described and a 22.7 kDa HSP, respectively. Genes CaHS3, CaHS4 and CaHS5 showed similar transcriptional behavior in all the *desi*

Table 1: Genes useful as expression markers to identify putative heat-tolerant chickpea genotypes.

Genes	Identification on *Ca* genome	Description	Sequences of Primers	Fragment Size (bp)	Tm (°C)
CaHS1	Ca_00150.1	HSP 70 kDa, mitochondrial	5′GAAAGGACTAAGGCTCCGTGT3′ 3′GTGGCGTTACATCAAGGAGT5′	250	59
CaHS3	Ca_00350.1	HSP	5′TGCTGAGATGTATAAGGTAGCGG3′ 3′AGCCATTCCATAGTTTCTTCCACT5′	138	60
CaHS5	Ca_01618.1	Small HSP C2	5′AACCAACGCTGCTGAATGTG3′ 3′TGTAGGTGGCTCAAGTGCAT5′	154	60
CaHS10	Ca_04162.1	HSP 81-2	5′TGAGAAAACCATTGAGAAGGAGA3′ 3′TCCCAATCGTTGGTAAGACTC5′	246	58
CaHS12	Ca_05577.1	HSP 17.1 kDa class II	5′ACCTGCAGACGTGAAAGAGT3′ 3′TCACCATTTTCAGGCAACACAA5′	219	59
CaHS14	Ca_05696.1	HSP DNA domain J	5′TCATGTTTCGAGTAGGACACCG3′ 3′TGTCGAATCTCCACGCTTTCT5′	175	60
CaHS15	Ca_05862.1	Small HSP, chloroplasts	5′CCCAACCCAAAAGAAGAACCG3′ 3′GGCATGTCAAACCTCATCTTGT5′	250	59
CaHS16	Ca_06052.1	HSP chloroplasts	5′TGTCCCAAAAAGCAGGGTACT3′ 3′CATGGCAACTCTACAAGGCT5′	203	59
CaHS19	Ca_07422.1	HSP 21.7 kDa class VI	5′TGATCCTTCAGATGCATTTCCTC3′ 3′GTTGTTCTTCCCTGTTCCTGG5′	151	58
CaHS21	Ca_08713.1	HSP DNA domain J	5′AAGGTGATACGGTTGCTGGT3′ 3′GTGTTCCACTCACCTGACCC5′	165	60
CaHS22	Ca_08899.1	HSP cognate protein 80	5′TTGCAGAGAACAAGGAGGATT3′ 3′TCACTCTGGCCTTCCTTCATC5′	190	58
CaHS24	Ca_09627.1	Activator of HSP 90 kDa	5′TTTCCGATGAGAACGCCGAT3′ 3′TCTTCTTCCCCACAACTGCC5′	237	59
CaHS26	Ca_09854.1	HSP	5′TGAAGGATCAAGGCAACGAGT3′ 3′AGCATCGTCGAGAGCTTTGT5′	164	59
CaHS37	Ca_11660.1	HSP 22.7 kDa class IV	5′CAGACCGTTTCCCTGATCCA3′ 3′TCCTTCCAGTCCACTCTGGC5′	106	60
CaHS38	Ca_11856.1	HSP 17.4 kDa class III	5′ATGACACAAGTGACAGTTGAAGA3′ 3′GAGGTGCTCTTCTCTCCAGTC5′	121	59
CaHS41	Ca_12998.1	Small HSP, chloroplasts	5′TGGCTGGAGATGAAGCAAGC3′ 3′TGCACTGTCCTAGCTACTGG5′	121	60
CaHS43	Ca_13382.1	HSP of factor HSF8	5′GAATGGAGCAACGGCAACAG3′ 3′CGCTTTTTGCTGACTTCGGT5′	130	60
CaHS46	Ca_13482.1	HSP	5′ATCGTGTGGAACGTAGCAGT3′ 3′CAGTGACAGTGAGAACACCA5′	111	59
CaHS48	Ca_13484.1	HSP	5′GGAGATTCAGATTGCCTGAGA3′ 3′CGATTGACTTGACATCAGGCTTC5′	120	59
CaHS53	Ca_15578.1	HSP 15.7 kDa, peroxisomal	5′TGGCTGAGACTATCTTTGGGC3′ 3′TGGGGATTCAAGCCAATCCAA5′	101	60
CaHS56	Ca_17398.1	HSP 18.5 kDa class I	5′TGTGGGGTGGAAGGAAACTC3′ 3′CATTTGAGCATCCTGAGGCA5′	202	60
CaHS58	Ca_17720.1	HSP factor HSF24	5′CCGGCACCGTTTTTAACCA3′ 3′GTGTTGAGTTGACGAACAAAGC5′	182	59
CaHS59	Ca_18155.1	HSP	5′CGGGTGGACTGGAAGGAAAC3′ 3′ACCACTGCTACGTTCAACCC5′	180	60
CaHS63	Ca_18920.1	HSP	5′ATCATCTGGCTGCGGAACTT3′ 3′TGTCTCTCTTCACCCCTCCAT5′	123	60

Table 2: Response classification, number of differential genes expressed and Heat Tolerance Index (TI) for *desi* and *kabuli* chickpea.

Type	Genotype	Classification[1]	Num. of differential genes	Average value per class	TI[2]
Desi	ICC 13020	T	33	Mean of T	0.62
	ICC 10259	T	31	30.5	0.75
	Annigeri	T	29		0.61
	ICC 4958	T	29		0.33
	ICC 96029	MT	26	Mean of MT	0.38
	ICC 5780	MT	26	23.8	0.42
	ICC 6671	MT	26		0.45
	El Patrón	MT	24		0.46
	ICC 3287	MT	24		0.22
	ICC 1282	MT	19		0.10
	Lerma	S	16	Mean of S	0.20
	San Antonio 05	S	16	11.5	0.15
	Line 123	S	10		-0.20
	ICC 2173	S	10		-0.16
	ICC 1882	S	9		-0.02
	ICC 7537	S	8		-0.07
Kabuli	Blanco Sinaloa 92	T	31	Mean of T	0.98
	ICCV2	T	31	28.4	0.51
	Tequi Blanco 95	T	27		0.67
	CUGA 08-743	T	27		0.55
	CUGA 08-1210	T	25		0.60
	Mazocahui	T	26		0.37
	HOGA 021	MT	23	Mean of MT	0.23
	JAMU 96	MT	21	20.7	0.30
	HOGA 067	MT	18		0.21
	HOGA 2004-20-6	S	12	Mean of S	-0.12
	Combo-751	S	11	10.5	-0.02
	Costa 04	S	10		-0.14
	Blancoson	S	9		-0.39

[1]T= tolerant, MT= moderately tolerant, S= susceptible.

[2]Calculated on the basis of adding expression values of individual responsive genes.

genotypes, probably because they share the same domain: a HSP of 20 kDa. Genes CaHS2 and CaHS18 were induced in most of the genotypes under the stress condition. The induced expression of the CaHS41 gene, which contains a HSP20 domain, was observed in all *kabuli* genotypes. The response of the rest of the genes was similar to that shown by *kabuli*; however, the expression of some genes was different between chickpea types: CaHS9 had a higher induction in the *desi* and CaHS53 in the *kabuli*.

Overall, the heat stress condition induced the transcription of the studied genes; *desi* genotypes showed higher levels of expression (Fig 1 and 3), as well as a higher number of induced genes compared to those in the *kabuli* genotypes. The latter could be explained by the fact that *desi* materials, being more resilient and less domesticated than *kabuli* types, have maintained greater genetic diversity and diverse heat tolerance mechanisms. Thus, although the differences observed in the expression profiles are clear and allow us to classify each chickpea genotype into tolerance classes by plotting the first versus second discriminant and on the same manner classifies *desi* and *kabuli* types (Fig 2), it is also important to establish that these differences, although significant, do not imply the absence of tolerance mechanisms in the *kabuli* type, since we also identified five heat stress-tolerant genotypes in this type (Fig 3).

Selection of *desi* and *kabuli* chickpea genotypes tolerant to heat stress

Previous research suggests that genotypes that induce the expression of a higher number of minor effect genes are those with a greater potential for tolerance to heat stress (Barnabás *et al.,* 2008; Porch and Hall, 2013). The cluster analysis (Fig 3) grouped the materials according to the total number of induced genes and this coincides with the tolerance reported in the field under heat conditions during the seed filling stage (>32°C); for *desi* and *kabuli* chickpea three groups were obtained: 1, genotypes with high potential tolerance to heat stress; 2, moderately tolerant genotypes; and 3, susceptible genotypes (Table 2, Fig 3). Regarding the *desi* genotypes, these outstanding were ICC 10259, ICC 13020, ICC 4958 and Annigeri; moderately tolerant

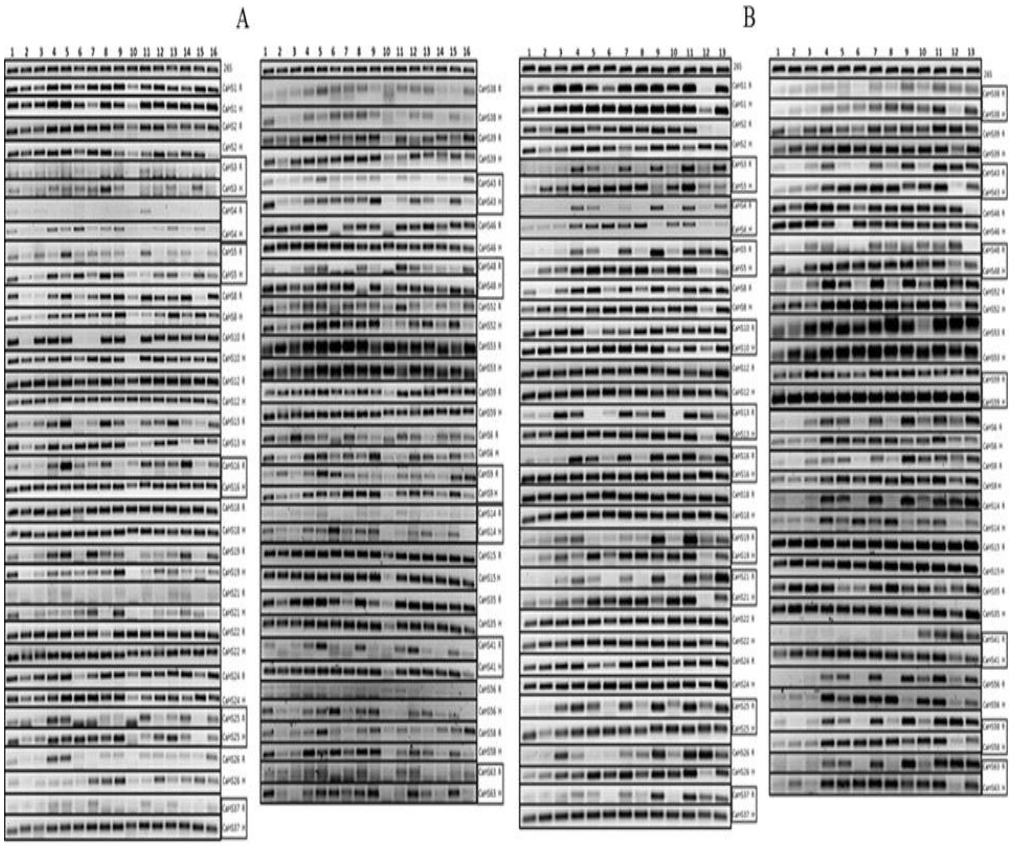


Fig 1: Profiles of expression of individual genes of *desi* and *kabuli* chickpea under two temperature treatments. Each line shows the expression levels of a gene from each chickpea genotype under normal (i.e. CAHS1R) and high temperature (i.e. CAHS1H) conditions. Gene identification is shown on the right side and corresponds to the description in Table 1. The numbers at the top show the chickpea genotypes. Squares indicate the genes that are mostly expressed under stress conditions. Panel A shows the *desi* and panel B the *kabuli* genotypes.

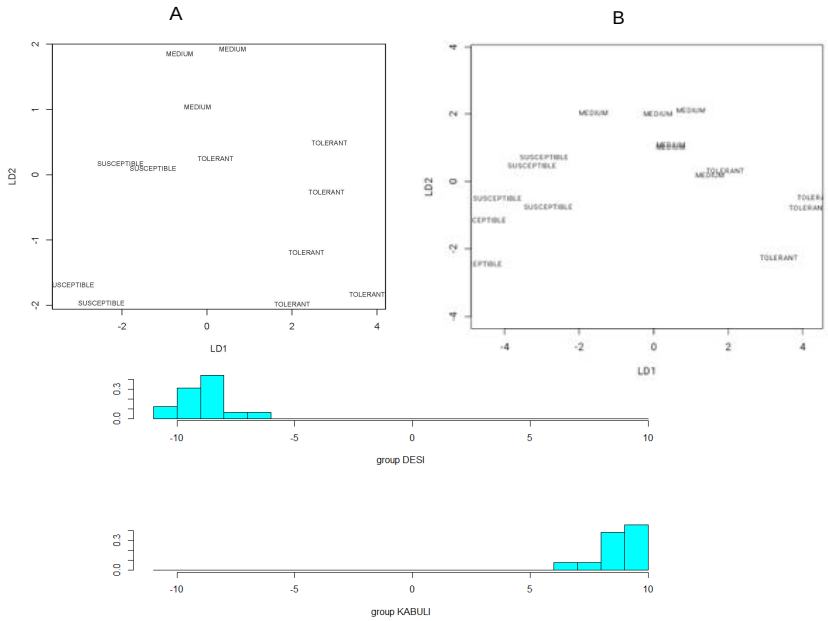


Fig 2: Discriminant analysis based on the expression levels of all genes studied. The first two discriminants classify into tolerance classes the *desi* (panel A) and *kabuli* (panel B) chickpea genotypes. This analysis applied to the chickpea types (panel C) perfectly separates both groups.

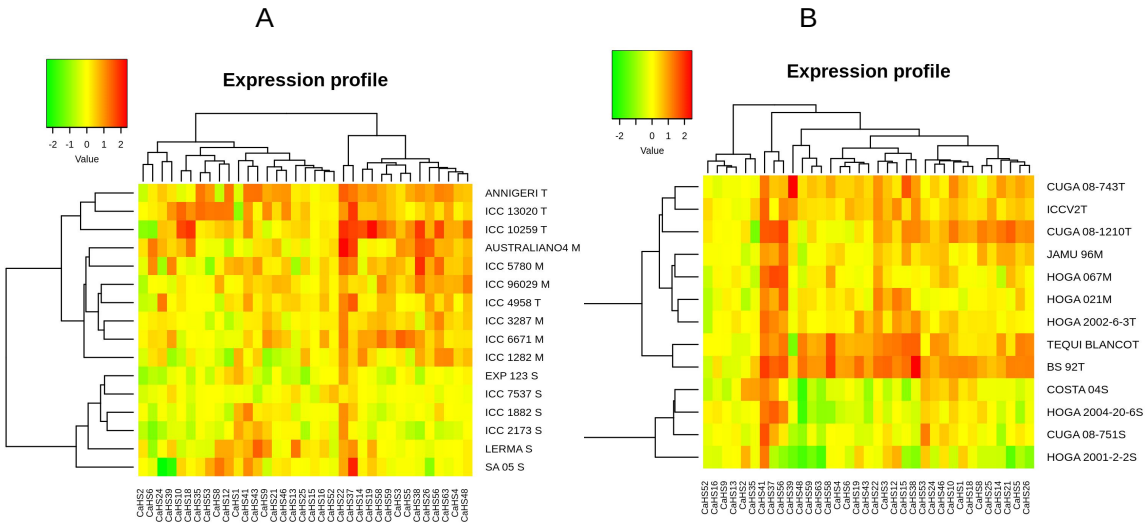


Fig 3: Heat maps showing in color-code the expression profiles of *desi* (A) and *kabuli* (B) chickpea genotypes. The upper dendrogram shows the similarity relationships of the genes based on their level of expression. On the left side, the dendrogram shows the similarity relationships of the genotypes based on their expression profile. The genotypes with predominance of red color (induced genes) are putative heat tolerant.

genotypes were ICC 96029, ICC 5780, ICC 6671, ICC 3287, ICC 1282 and El Patrón; susceptible genotypes were ICC 2173, line 123 and ICC 1882. Some of these materials have been reported as tolerant to heat stress due to their physiological and phenological responses; Krishnamurthy *et al*. (2011) reported ICC 4958 as tolerant, its response mechanism is early flowering and high seed yield. Cultivar Annigeri has also been reported as tolerant due to its high cell membrane stability against heat stress (Srinivasan *et al*. 1996). The *kabuli* genotypes were grouped as follows: group 1, Mazocahui, ICCV 2, Blanco Sinaloa 92, Tequi Blanco 95, Combo-743 and CUGA 08-1210; group 2, HOGA 021, JAMU 96, HOGA 067; group 3, HOGA 2004-20-6, CUGA 08-751, Costa 2004 and Blancoson (Fig 3). ICCV 2 has also been reported as tolerant because of its physiological characteristics, showing early flowering as the main response against heat stress (Kumar and Abbo, 2001).

The Tolerance Index (TI) based on the adding of individual gene expression levels (Table 2) describes the chickpea genotypes in a similar way as the profiles of expression, since their values almost coincided with the classification of tolerant, moderately tolerant and susceptible. Therefore, it is proposed that both strategies could be used as complementary criteria for the identification of chickpea genotypes tolerant to high temperatures.

CONCLUSION

The methodology used in this research allowed for the classification of *kabuli* and *desi* chickpea genotypes into tolerant, moderately tolerant and susceptible. Twenty-four genes were identified that induced their transcription under heat stress, ten of which can be useful to study both types of chickpea, eight were exclusive of *kabuli* and six of *desi* type.

Desi genotypes with potential heat tolerance are ICC 10259, ICC 13020, ICC 4958 and Annigeri; and the *kabuli* Mazocahui, ICCV 2, Blanco Sinaloa 92, Tequi Blanco 95, Combo-743 and CUGA 08-1210. Results suggests that *desi* chickpea has higher heat tolerance potential than *kabuli*; however, the methodology also allowed the identification of tolerant *kabuli* genotypes. We will integrate this information into the local chickpea improvement program for the development of new varieties tolerant to heat stress.

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