Journal of Genetics (2021)100:71
https://doi.org/10.1007/s12041-021-01317-y

RESEARCH ARTICLE

Characterization and differential expression of sucrose and starch metabolism genes in contrasting chickpea (*Cicer arietinum* L.) genotypes under low temperature

KAMAL DEV SHARMA*, GAURAV PATIL and ASHA KIRAN

Department of Agricultural Biotechnology, CSK Himachal Pradesh Agricultural University, Palampur 176 062, India
*For correspondence. E-mail: kml1967@rediffmail.com.

Received 14 December 2020; revised 22 May 2021; accepted 1 June 2021

Abstract. Low temperature (LT) causes significant yield losses in chickpea (*Cicer arietinum* L.). The sucrose starch metabolism is associated with abiotic-stress tolerance or sensitivity in plants. The changes in sugars and starch contents under LT in chickpea have already been studied, however, no information is available on LT-induced alterations in transcription of carbohydrate metabolic pathway genes in chickpea. To understand the differences in the regulation of sucrose and starch metabolism under LT, the expression of sucrose and starch metabolism genes was studied in leaves of cold-sensitive (GPF2) and cold-tolerant (ICC 16349) chickpea genotypes. The mRNA sequences of chickpea genes were retrieved from the public databases followed by confirmation of identity and characterization. All the genes were functional in chickpea. Between the two paralogues of cell wall invertase, cell wall invertase 3×2 (*CWINx2*) was the truncated version of cell wall invertase 3×1 (*CWINx1*) with the loss of 241 bases in the mRNA and 67 amino acids at N terminal of the protein. Comparison of expression of the genes between control (22°C day / 16°C night) and LT treated (4°C; 72 h) plants revealed that granule bound starch synthase 2 (*GBSS2*) and β-amylase 3 (*BAM3*) were upregulated in ICC 16349 whereas sucrose phosphate synthase 2 (*SPS2*), *CWINx1*, *CWINx2* and β-amylase 1 (*BAM1*) were downregulated. In contrast to this, *SPS2*, *CWINx1*, *CWINx2* and *BAM1* were upregulated and *GBSS2* downregulated in GPF2 under LT. The gene expression data suggested that *UGPase*, *CWINs*, *GBSS2* and *BAM3* are important components of cold-tolerance machinery of chickpea.

Keywords. chickpea; cell wall invertase; granule bound starch synthase; gene expression; low temperature; sucrose phosphate synthase

Introduction

Chickpea, the second most important food grain legume worldwide (Gaur *et al*. 2008; Varshney *et al*. 2013; Kohli *et al*. 2014), is a vital source of dietary proteins in semi-arid tropics (Sharma and Lavanya 2002; Tesfaye *et al*. 2006; Muehlbauer and Rajesh 2008; Kaloki *et al*. 2019; Sofi *et al*. 2020; Varol *et al*. 2020). It occupies an area of 17.81 million hectares with a total production of 17.1 million tons and productivity of 9651 hg/ha (FAOSTAT 2020, http://www.fao.org/faostat/en/#data/QCL). Chickpea productivity is affected by abiotic stresses such as low temperature (LT), high temperature and drought (Ruelland *et al*. 2002; Jha *et al*. 2014; Kohli *et al*. 2014; Garg *et al*. 2015). Chickpea evolved in the warmer climates of the Mediterranean region and is sensitive to cold stress. Chilling temperatures (below 10°C) affect its yield and productivity, more so, if chilling stress coincides with the reproductive stage (Kaur *et al*. 2008, 2009; Kumar *et al*. 2010, 2011, 2013; Rani *et al*. 2019). Cold stress affects normal growth and development of plants (Chinnusamy *et al*. 2007; Bhandari *et al*. 2017; Kiran *et al*. 2019) and at reproductive stage, it leads to flower/pod abortion and impedes seed filling (Kaur *et al*. 2008, 2009; Kumar *et al*. 2010, 2011, 2013; Kiran *et al*. 2019). The impact of cold stress on carbohydrate metabolisms has been studied in crops such as *Arabidopsis* (Dèjardin *et al*. 1999; Jung *et al*. 2003; Nägele and Heyer 2013), potato (Oufir *et al*. 2008), tea (Yue *et al*. 2015) and chickpea (Kaur *et al*. 2008, 2009; Kumar *et al*. 2011). Carbohydrate perturbation is one of the major phenomena in

Supplementary Information: The online version contains supplementary material available at https://doi.org/10.1007/s12041-021-01317-y.

Published online: 30 September 2021

plants under cold stress (Sharma and Nayyar 2016). Changes in the expression of various sucrose and starch metabolism associated genes such as uridine diphosphate glucose pyrophosphorylase (*UGPase*) (Sowokinos *et al*. 1997; Ciereszko *et al*. 2001; Meng *et al*. 2007), sucrose phosphate synthase (*SPS*) (Wang *et al*. 2013), invertases (Deryabin *et al*. 2005; Wang *et al*. 2013), granule-bound starch synthase (*GBSS*) (Wang *et al*. 2006) and beta amylase (*BAM*) (Seki *et al*. 2001; Kaplan and Guy 2004; Kaplan *et al*. 2006; Liu *et al*. 2013) are observed in other crops under LT stress, whereas no such information is available for chickpea.

Small number of chickpea genes, expressing differentially under LT stress have been identified using the techniques such as microarray, differential display reverse transcriptase polymerase chain reaction (DDRT PCR) and cDNA AFLP. Fifty-four genes that expressed differentially under LT in a cold-sensitive genotype were identified using microarray (Mantri *et al*. 2010) whereas cDNA AFLP identified 102 transcript-derived fragments that expressed differentially in a cold tolerant genotype under LT (Dinari *et al*. 2013). Between the cold-tolerant and cold-sensitive genotypes under LT, 15 genes were expressed differentially (Mantri *et al*. 2010). Cold acclimation, a phenomenon where gradual decrease in temperature over a period of time induces cold-tolerance, was associated with increased expression of antioxidants genes (Kazemi-Shahandashti *et al*. 2014). In anthers of a cold-tolerant genotype of chickpea, genes of carbohydrate metabolism and proline transport were upregulated under LT whereas no such increase was observed in the cold-sensitive genotype (Sharma and Nayyar 2014; Kiran *et al*. 2021).

While phenomena of LT sensitivity is understood fairly well, the mechanisms governing LT tolerance including regulation of genes under LT tolerance are largely unknown in legume crops including chickpea. Earlier studies point towards the role of carbohydrate metabolism in LT tolerance in plants (Strand *et al*. 1997; Yamaguchi-Shinozaki and Shinozaki 2006; Oufir *et al*. 2008; Sharma and Nayyar 2014; Thakur *et al*. 2020). In leaves of cold tolerant chickpea genotype under LT, an increase in carbohydrate content and their associated enzymes activity was observed (Kaur *et al*. 2009; Kumar *et al*. 2011). In cold-sensitive chickpea genotype GPF2, LT decreased the levels of starch, sucrose and soluble sugars in leaves (Kaur *et al*. 2008). Comparison of metabolite contents in leaves of cold-tolerant (ICC 16348 and ICC 16349) and cold-sensitive (GPF2 and PBG1) genotypes of chickpea revealed that amounts of total sugars, reducing sugars, starch and activities of carbohydrate enzymes sucrose sythase, inveratase and β-amylase under LT were higher in the cold-tolerant genotypes compared to the cold-sensitive ones (Kaur *et al*. 2009; Kumar *et al*. 2011). The knowledge of LT-induced alterations in the transcription of genes encoding enzymes of carbohydrate metabolism is expected to enhance the understanding of the mechanisms by which LT tolerant and LT sensitive chickpea plants modified the contents of various carbohydrates under LT stress as observed by Kaur *et al*. (2009) and Kumar *et al*. (2011). Sucrose–starch metabolism is also important for maintenance of photosynthesis in source tissues as well as for induction of cold-tolerance in crops such as *Arabidopsis* (Strand *et al*. 2003; Klotke *et al*. 2004), alfalfa (Mo *et al*. 2011), wheat (Savitch *et al*. 1997, 2000) and chickpea (Kaur *et al*. 2009; Kumar *et al*. 2011). Evidently, changes in sucrose–starch metabolism under LT might be affecting photosynthesis in chickpea.

Since, LT perturbs sugar–starch metabolism differentially in the cold-tolerant and cold-sensitive genotypes of chickpea, the present study aims to analyse changes in the expression of sugar and starch metabolism genes in a cold-tolerant (ICC 16349) and a cold-sensitive (GPF2) genotypes of chickpea under LT and normal temperature conditions. The genome of the chickpea has been sequenced (Jain *et al*. 2013; Varshney *et al*. 2013) and was used to retrieve and characterize sugar and starch metabolism genes from chickpea genome for expression analysis.

Materials and methods

Plant material and growth condition

The seeds of the cold-tolerant (ICC 16349) and cold-sensitive (GPF2) chickpea genotypes were sown in plastic pots (4″ diameter pots; two plants per pot) filled with soil. After germination, the pots were shifted to a growth chamber (Bhanu Biotech, Delhi, India) at 22±1°C / 16±1°C day/night temperature (normal temperature (control)), 50–70% relative humidity, and 16 h photoperiod, 15000 lux (luminous flux per unit area) light intensity produced by cool white fluorescent lamps until the plants had 3–4 leaves. For cold treatment, plants growing under NT were exposed to 4°C for a period of 72 h in cold chambers. Except temperature, the rest of the conditions were kept similar to control. The leaves of cold-treated and control plants were sampled in three replicates from each genotype for use in subsequent gene expression analysis.

Characterization of mRNA, deduced proteins and primer development

The nucleotide sequences of the 10 genes (sucrose synthesis: UDP-glucose pyrophosphorylase (UGPase), sucrose phosphate synthase 2 (*SPS2*), sucrose transport and catabolism: cell wall invertase 3×1 (*CWIN3x1*), cell wall invertase 3×2 (*CWIN3x2*), starch synthesis: ADP-glucose pyrophosphorylase (*AGPase*), soluble starch synthase 1 (*SSS1*), granule-bound starch synthase 2 (*GBSS2*) and starch catabolism: isoamylase 3, beta-amylase 1 (*BAM1*) and beta-amylase 3 (*BAM3*)) related to sugar and starch metabolism were retrieved from The National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/). The cell wall invertases not only

Table 1. Primer sequences of carbohydrate genes.

	Gene name	Primer sequence (5′–3′)
1	UDP glucose pyrophosphorylase	Forward: CCTGGTCATGGGGATGTCTT Reverse: GTGCCACCCTTAACATCAGC
2	Sucrose phosphate synthase 2	Forward: GGCGCTTTGAATGTACCGAT Reverse: TCCCCACTGCTCCTCAATTT
3	Cell wall invertase 3×1	Forward: ATGAACGATCCAAATGCCCC Reverse: CTGAGCCTGACCAACAACTG
4	Cell wall invertase 3×2	Forward: GAGACCCTTCAACTGCATGG Reverse: CCACAGATGTATCCACCCCA
5	ADP-glucose pyrophosphorylase	Forward: AAGCCTGCTGTTCCAATTGG Reverse: CCCTGGAGTTTGAGTTGCTG
6	Soluble starch synthase 1	Forward: GGCGTCAAAGTATCGTCCAC Reverse: CCGATCGGCTGTAACAATGG
7.	Granule-bound starch synthase 2	Forward: CTGCCGGTGTATCTGAAAGC Reverse: AAACCGGCTGCAAAGATGTT
8	Isoamylase 3	Forward: ACACAACTTCATTCCGTGGC Reverse: CGACACAGTACACTTGCGAG
	Beta-amylase 1	Forward: CAGTGCCGTCAGAAACAACA Reverse: TAGATCTGGCCGGATTCCAC
10	Beta-amylase 3	Forward: AGTGCTGGAGTTGAAGGTGT Reverse: AGGAGGTAGAGGAATGCTGC

transport sucrose but also catabolize it to glucose and fructose. The identity of each gene was established using the 'blastn' tool of NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). The identity of the genes was confirmed by analysis of the deduced proteins by 'blastp' (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) tool of NCBI. The identity, biological function and molecular function of the proteins were deduced from the KEGG pathway (http://www.genome.jp/kegg/). Nucleotide-coding sequences (cds) containing open-reading frame (ORFs) of respective genes were also retrieved (https://www.ncbi.nlm.nih.gov/orffinder/). Conserved domains, active sites and binding sites of the deduced enzymes were searched using InterPro tool of EMBL-EBI (https://www.ebi.ac.uk/interpro) and NCBI conserved domain search tool (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

The ORFs of the genes were used for primer designing. The primers were designed by using the Batch Primer3 software (http://probes.pw.usda.gov/cgi-bin/batchprimer3/batchprimer3.cgi) and were custom synthesized from Integrated DNA technologies (Thermo Fisher Scientific, USA).

RNA isolation and cDNA synthesis

Total RNA was isolated from leaf tissues using the Nucleospin RNA extraction kit (Macherey-Nagel, Germany) as per the manufacturer's instructions. Traces of DNA were removed from isolated RNA by DNase I treatment. For DNase I treatment, RNA (4.0 μL, 2.0 μg), 10× reaction buffer with $MgCl_2$ (1.0 μL), DNase I (RNase-free, 1.0 μL, 1 U; Thermo Fisher Scientific USA) and nuclease-free water (4.0 μL) was added to each RNase-free tube followed by incubation at 37°C for 30 min. One μL ethylenediaminetetraacetic acid (EDTA; 50 mM) was added to each tube and contents were incubated at 65°C for 10 min. The quantity and quality of RNA was assessed by nanodrop (Thermo-Scientific Model) and on 1% formaldehyde agarose gel (MOPS), respectively. Hundred ng of template RNA (2.5 μL) was used to synthesize cDNA by using 100 μM oligo $(dt)_{18}$ primer in 11 μL DEPC-treated water at 65°C for 5 min. The tubes were placed in ice bath before adding the cDNA synthesis mix, including 4 μL of reaction buffer, 1 μL of RiboLock RNase inhibitor (20 U/μL), 2 μL of dNTP Mix (10 mM), M-MuLV reverse transcriptase (20 U/μL) (Thermo-scientific, USA) and DEPC-treated water.

Reverse transcriptase PCR (RT-PCR)

cDNAs of genes were used as templates for semiquantitative RT-PCR. The PCR reaction mix contained 2 μL of diluted (1:25) cDNA, 5 μL coloured buffer (Promega), 1.5 μL $MgCl_2$ solution (25 mM), 2.5 μL dNTP (2 mM), 1 μL each of gene specific reverse and forward primers (10 μM), 0.125 μL Go Tag flexi DNA polymerase (5 U/μL; Promega) in a final volume of 25 μL with double-distilled water and gene-specific primers (see table 1 for sequences of primers). The PCR programme included: initial denaturation at 94°C for 3 min followed by 36 cycles of denaturation at 94°C for 30 s, annealing at 53–56°C for 30 s and extension at 72°C for 30 s. Two reference genes, i.e. Clathrin adaptor complexes (*CAC*) and ATP-binding cassette transporter (*ABCT*) were used for normalization of gene expression data. Negative control (without template cDNA) was also used to confirm absence of genomic DNA. Images of the RT-PCR ethidium bromide-stained agarose gels were taken with a Gel

documentation system (Bio-Rad USA) and band intensity was expressed as relative absorbance units. The mean of relative absorbance of *CAC* and *ABCT* was used to normalize the gene expression data. The experiment has three biological and three technical replicates.

Results

Characterization of mRNAs and deduced proteins

***UDP-glucose pyrophosphorylase*:** The mRNA of UDP-glucose pyrophosphorylase (*UGPase*) was 1782 bases long with an open-reading frame (ORF) of 1428 bases, 5′UTR of 87 bases and relatively long 3′UTR of 267 bases (table 2; see table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/ for ORF, 5′UTR and 3′UTR). The GC content of ORF was 42%, and the enzyme UDP glucose pyrophosphorylase had 475 amino acids (table 3; table 2 in electronic supplementary material). The conserved domain of the enzyme had amino acids ranging from 92 to 381, and the active and binding sites were at amino acids 92–94, 105, 140, 168, 195, 197–198, 226, 262–264, 277, 299 and 366.

***Sucrose phosphate synthase 2*:** The mRNA of *sucrose phosphate synthase* 2 (*SPS2*) gene was comprised of 3871 bases with ORF of 3129 bases, 3′UTR of 155 bases and unusually long 5′UTR (587 bases) (table 2; table 3 in electronic supplementary material). GC content of ORF was 41% and SPS2 was comprised of 1042 amino acids (table 3; table 4 in electronic supplementary material). The enzyme had three domains: N-terminal sucrose synthase domain (167–396 amino acids), central Glyco_trans_1 domain (glycosyl transferase, 475–647 amino acids) and C-terminal sucrose phosphate synthase domain (758–991 amino acids) (table 3). The active sites of the enzyme were at 764–766, 803, 950, 968 and 973 amino acids.

***Paralogues of cell wall invertase: similarities and differences between genes and deduced proteins*:** The mRNAs of the two paralogues of chickpea cell wall invertase genes (*CWINx1* and *CWINx2*) were also characterized. The mRNA of *CWINx1* was comprised of 2102 bases with 143 bases long 5′UTR and 210 bases long 3′UTR (table 2; table 5 in electronic supplementary material). The length of the ORF was 1749 bases. GC content of ORF was 37% (table 2) and CWINx1 was comprised of 582 amino acids (table 3; table 6 in electronic supplementary material). The mRNA of *CWINx2* was comprised of 1861 bases with 1548 bases long ORF (table 2; table 7 in electronic supplementary material). The 5′UTR was 103 bases long whereas 3′ UTR region was 210 bases long. GC content of ORF of *CWINx2* was 37% (table 2). The CWINx2 was comprised of 515 amino acids (table 3; table 8 in electronic supplementary material).

The mRNAs of two paralogues of *CWIN* had 87% similarity to each other with no similarity in the 5′UTRs, 88% similarity in the ORFs and 100% similarity in the 3′UTRs (figure 1). In the ORFs of two mRNAs, the 201 bases at 5′ region were present in *CWINx1* and absent in *CWINx2* indicating a deletion at 5′end in *CWINx2*. However, first 59 bases of 5′UTR of *CWINx2* were part of the ORF of *CWINx1* suggesting duplications, deletions and chromosomal rearrangements as mechanisms of evolution of new variant (*CWINx2*) from *CWINx1* (figure 1).

The deduced protein sequences of CWINx1 and CWINx2 were 88% similar. The CWINx1 and CWINx2 proteins were similar to each other except for first 67 amino acids which were present in CWINx1 but absent in CWINx2 (figure 2). Thus, the CWINx2 was a truncated version of CWINx1 with loss of 67 amino acids. Conserved domain search revealed that first 52 amino acids of CWINx1 were not part of the conserved domain of the enzyme. Similarly, amino acids after 528 were not part of conserved domain (table 3). For CWINx2, amino acids from 1 to 461 were part of conserved domain of the enzyme. Thus, conserved domain of CWINx1 (476 amino acids) was longer by 15 amino acids as compared to CWINx2 (461 amino acids). Both the paralogues were comprised of two domains: Glyco_hydro_32_N at 53-371 amino acids in CWINx1 and at 1-304 in CWINx2 and Glyco_hydro_32_C at 374-567 amino acids in CWINx1

Table 2. Characterization of mRNAs of 10 carbohydrate metabolism genes in chickpea.

	Gene name	Gene ID	Length of mRNA (bases)	Length of 5′UTR (bases)	Length of ORF (bases)	Length of 3′UTR (bases)	GC content of ORF
1	UDP glucose pyrophosphorylase	LOC101490076	1782	87	1428	267	42%
2	Sucrose phosphate synthase 2	LOC101499894	3871	587	3129	155	41%
3	Cell wall invertase 3×1	LOC101500539	2102	143	1749	210	37%
4	Cell wall invertase 3×2	LOC101500539	1861	103	1548	210	37%
5	ADP-glucose pyrophosphorylase	LOC101504730	1809	82	1578	149	40%
6	Soluble starch synthase 1	LOC101512422	2395	154	1929	312	43%
7	Granule-bound starch synthase 2	LOC101489359	2806	248	2286	272	42%
8	Isoamylase 3	LOC101496876	3062	267	2343	452	41%
9	Beta-amylase 1	LOC101508440	2012	40	1722	250	46%
10	Beta-amylase 3	LOC101494122	1976	46	1638	292	36%

UTR, untranslated region; ORF, open reading frame.

Table 3. Characterization of deduced protein sequences of carbohydrate metabolism enzymes in chickpea.

	Enzyme	Protein length (amino acids)	Number of conserved domains	Name of conserved domains	Amino acid residues of conserved domains	Amino acid residues of active sites
1	UDP glucose pyrophosphorylase	475	One	UDP glucose pyrophosphorylase	92–381	92–94, 105, 140, 168, 195, 197–198, 226, 262–264, 277, 299 and 366
2	Sucrose phosphate synthase 2	1042	Three	N-terminal sucrose synthase domain	167–396	764–766, 803, 950, 968 and 973
				Central glyco_trans_1 domain (glycosyl transferase)	475–647	
				C-terminal sucrose phosphate synthase domain	758–991	
3	Cell wall invertase 3×1	582	Two	Glyco_hydro_32_N	53–371	53–66, 63, 188 and 242
				Glyco_hydro_32_C	374–567	
4	Cell wall invertase 3×2	515	Two	Glyco_hydro_32_N	1–304	NI
				Glyco_hydro_32_C	307–500	
5	ADP-glucose pyrophosphorylase	525	One	NTP_transferase	94–371	NI
6	Soluble Starch synthase 1	642	Two	Starch_synth_cat_dom	129–387	NI
				Glyco_trans_1	443–579	
7	Granule-bound starch synthase 2	761	Two	Starch synthase catalytic domain	271–513	NI
				Glycosyl transferase domain	571–714	
8	Isoamylase 3	780	Four	GlgX_Isoamylase_N_E_set	107–256	442, 444, 481, 553
				Glyco_hydro_13_N	108–206	
				Glyco_hydro_13_cat_dom	256–663	
				Alpha-amylase	277–384	
9	Beta-amylase 1	573	Three*	*Glyco_hydro_14_CS (O-glycosyl hydrolase family 14 conserved site),	188–286	NI
				β-amylase_1	188–196	
				β-amylase_2	276–286	
10	Beta-amylase 3	545	One*	Glyco_hydro_14_CS	169–177	

*Conserved sites. NI, no information

and at 307–500 in CWINx2. The active site of CWINx1 was at 53–66, 63, 188 and 242 amino acids whereas no active sites for CWINx2 were revealed by the software. The β-sandwich sites were detected at 96, 102, 103, 106, 313, 334, 351, 353, and 3356 amino acid residues for CWINx1 and at 29, 35–37, 39, 246, 267–268, 284, 286–287 and 289 residues for CWINx2.

***ADP-glucose pyrophosphorylase*:** The mRNA of ADP-glucose pyrophosphorylase (AGPase) was 1809 bases in length with 82 bases long 5′UTR, 1578 bases long ORF (GC content = 40%) and 149 bases long 3′UTR (table 2; table 9 in electronic supplementary material). The size of the AGPase was 525 amino acids and the enzyme had only one domain (NTP_transferase) at 94–371 amino acids (table 3; table 10 in electronic supplementary material).

***Soluble starch synthase 1*:** The soluble starch synthase 1 (*SSS1*) mRNA had 2395 bases with 154 bases long 5′UTR, 1929 bases long ORF and relatively longer 3′UTR (312 bases) (table 2; table 11 in electronic supplementary material). The GC content in ORF was 43%. The enzyme soluble starch synthase 1 was 642 amino acids in length and had two domains (Starch_synth_cat_dom at 129-387 amino acids and Glyco_trans_1 at 443-579 amino acids) (table 3; table 12 in electronic supplementary material).

***Granule-bound starch synthase 2*:** The mRNA of the granule-bound starch synthase 2 (*GBBS2*) was comprised of 2806 bases with 2286 bases long ORF, 248 bases long 5′UTR and 272 bases long 3′UTR (table 2; see table 13 in electronic supplementary material for ORF, 5′UTR and 3′UTR). GC

```
CWINx1   ACAAAGTGACAGATCAACTATAAATAAATATATTAACCATACGCTCTGATTTAATTTACA   60
CWINx2   ------------------------------------------------------------   0

CWINx1   TATATAAAGTCCCTGATCAAACTTAAATCAAAACCCATCTTGCTTTTGTTGACCTTCCTC   120
CWINx2   ------------------------------------------------------------   0

CWINx1   TTCTCCTTTCATGCTTGTGCATCATGAAAAACTTTGCAGCATCACTATTACTTGTGATAA   180
CWINx2   ------------------------------------------------------------   0

CWINx1   TTTCTTTTGTGATAATTTCTTTTCTGTTAAACAATGGAATTAAGGCATCAACACATAGTA   240
CWINx2   ------------------------------------------------------------   0

CWINx1   ACAGTGATGATCATATCAAGTACATGCTACCTGATGAACAACCTTACAGAACTTCTTATC   300
CWINx2   --------------------------------GATGAACAACCTTACAGAACTTCTTATC   28
                                          ****************************

CWINx1   ATTTTCAGCCCCCACAATATT------------------------------GGATGAAC   329
CWINx2   ATTTTCAGCCCCCACAATATTGGATGAACGAAAATTGATAGTGGATGACTTCATTATGCA   88
         *********************

CWINx1   GATCCAAATGCCCCTATGTACTACAAAGGTGTTTACCACCTTTTCTACCAACATAATCCT   389
CWINx2   GATCCAAATGCCCCTATGTACTACAAAGGTGTTTACCACCTTTTCTACCAACATAATCCT   148
         ************************************************************

CWINx1   TATGCAGCAACATTTGGTGATAGGATTATATGGGCTCATTCAGTATCCTATGATCTCATA   449
CWINx2   TATGCAGCAACATTTGGTGATAGGATTATATGGGCTCATTCAGTATCCTATGATCTCATA   208
         ************************************************************

CWINx1   AGTTGGATTCATCTAAATCATGCTATTGAGCCAAGTGAGCCTTATGACATCAACAGTTGT   509
CWINx2   AGTTGGATTCATCTAAATCATGCTATTGAGCCAAGTGAGCCTTATGACATCAACAGTTGT   268
         ************************************************************

CWINx1   TGGTCAGGCTCAGCCACTATACTCCCAGGTGAAAAACCTGCTATTTTGTACACAGGAATT   569
CWINx2   TGGTCAGGCTCAGCCACTATACTCCCAGGTGAAAAACCTGCTATTTTGTACACAGGAATT   328
         ************************************************************
```

Figure 1. Base similarity in mRNAs of paralogues (*CWINx1* and *CWINx2*) of cell wall invertases of chickpea. The deletions were in the 5′ untranslated region and at the start of the ORF of the mRNAs of *CWINx2* as compared to *CWINx1*. The 3′ untranslated regions were similar suggesting the *CWINx2* is the truncated version of *CWINx1*.

```
CWINx1    MKNFAASLLLVIISFVIISFLLNNGIKASTHSNSDDHIKYMLPDEQPYRTSYHFQPPQYW    60
CWINx2    ------------------------------------------------------------    0

CWINx1    MNDPNAPMYYKGVYHLFYQHNPYAATFGDRIIWAHSVSYDLISWIHLNHAIEPSEPYDIN    120
CWINx2    -------MYYKGVYHLFYQHNPYAATFGDRIIWAHSVSYDLISWIHLNHAIEPSEPYDIN    53
                 **************************************************

CWINx1    SCWSGSATILPGEKPAILYTGIDKNKHQVQNLAFPKNLSDPFLREWEKHPQNPVMTSPSG    180
CWINx2    SCWSGSATILPGEKPAILYTGIDKNKHQVQNLAFPKNLSDPFLREWEKHPQNPVMTSPSG    113
          ************************************************************

CWINx1    VEKDNFRDPSTAWHGNDGKWRVIIGAQNGDEGKTILYQSDDFLNWTVNPNPFFATDDTGV    240
CWINx2    VEKDNFRDPSTAWHGNDGKWRVIIGAQNGDEGKTILYQSDDFLNWTVNPNPFFATDDTGV    173
          ************************************************************

CWINx1    CECPEFFPVYINSTNGVDTSVENQSVRHVLKISYIRIQHDYYFVGKYVSEKEKFTPDVEF    300
CWINx2    CECPEFFPVYINSTNGVDTSVENQSVRHVLKISYIRIQHDYYFVGKYVSEKEKFTPDVEF    233
          ************************************************************

CWINx1    KGTSSDLRFDYGKFYASKSFFDYAKNRRILWGWVNESDTTQDDIEKGWAGLQTIPRQVWL    360
CWINx2    KGTSSDLRFDYGKFYASKSFFDYAKNRRILWGWVNESDTTQDDIEKGWAGLQTIPRQVWL    293
          ************************************************************

CWINx1    DKSGKRLMQWPIEELENLRDKQISITEQKLESGSTLEVLGITASQVDVEVLFELPELESG    420
CWINx2    DKSGKRLMQWPIEELENLRDKQISITEQKLESGSTLEVLGITASQVDVEVLFELPELESG    353
          ************************************************************

CWINx1    EWLDPSEVDPQLLCSKQYESRSGKIGPFGLLALASKDLTEQTAVSFQIYRAPNRYICLMC    480
CWINx2    EWLDPSEVDPQLLCSKQYESRSGKIGPFGLLALASKDLTEQTAVSFQIYRAPNRYICLMC    413
          ************************************************************

CWINx1    SDQSRSSLRQDLDKTTYGTIFDIDANLKTISLRSLIDKSIIESFGDGGRACITSRVYPSL    540
CWINx2    SDQSRSSLRQDLDKTTYGTIFDIDANLKTISLRSLIDKSIIESFGDGGRACITSRVYPSL    473
          ************************************************************

CWINx1    AIEKDAHLYVFNNGSQSVVISKLNAWSMKQAEFSHEENISCA        582
CWINx2    AIEKDAHLYVFNNGSQSVVISKLNAWSMKQAEFSHEENISCA        515
          ****************************************
```

Figure 2. Amino acid similarity in deduced proteins (CWINx1 and CWINx2) of paralogues of cell wall invertase of chickpea. The deletions were in the N-terminus of the protein of CWINx2 as compared to CWINx1 confirming that the CWINx2 is the truncated version of CWINx1.

content of ORF of gene *GBBS2* was 42% (table 2) and GBBS2 had 761 amino acids (table 3; table 14 in electronic supplementary material). The enzyme comprised of two domains: starch synthase catalytic domain (271–513 amino acids) and glycosyl transferase domain (571–714 amino acids) (table 3). The homodimer interfaces were present at amino acid residues 467, 680, 685, 688 and 713 and the ADP binding pockets were at 284, 287, 581–582, 637–638, 643, 660 and 665.

***Isoamylase 3*:** The *Isoamylase 3* mRNA was 3062 bases in length with ORF of 2343 bases, 5′UTR of 267 bases and a long 3′UTR (452 bases) (table 2; table 15 in electronic supplementary material). The ORF had GC content of 41%. The enzyme isoamylase 3 was 780 amino acids in length with four domains, i.e. GlgX_Isoamylase_N_E_set (107-256 amino acids), Glyco_hydro_13_N (108-206 amino acids), Glyco_hydro_13_cat_dom (256-663 amino acids) and alpha-amylase (277-384 amino acids) (table 3; table 16 in electronic supplementary material). The active sites were present at 53–66, 63, 188 and 242 amino acids (table 3).

***Similarities and differences between beta amylase genes and deduced proteins*:** The mRNA of *beta-amylase 1* was 2012 bases long with ORF of 1722 bases, 5′UTR of 40 bases and 3′UTR of 250 bases (table 2; table 17 in electronic supplementary material). The GC content of ORF was 46% (table 2) and enzyme beta-amylase 1 had 573 amino acids (table 3; table 18 in electronic supplementary material). Beta-amylase 1 had three conserved sites: O-glycosyl

hydrolase family 14 conserved site (188–286 amino acids), β-amylase_1 site (188–196 amino acids) and β-amylase_2 site (276–286 amino acids). The *beta-amylase 3* mRNA was 1976 bases in length with a short 5′UTR (46 bases) and a long 3′UTR (292 bases) (table 2; table 19 in electronic supplementary material). The GC content of ORF was 36%. The beta-amylase 3 (size: 545 amino acids) had only one conserved site, O-glycosyl hydrolase family 14 conserved site at 169–177 amino acids (table 3; table 20 in electronic supplementary material).

The *beta-amylase 1* and *beta-amylase 3* mRNAs showed no sequence similarity suggesting unrelated origin of the two genes. The protein sequence similarity was also low (61%) and N-terminal 104 amino acids of beta-amylase 1 and 85 of beta-amylase 3 were dissimilar whereas at C-terminal, only 15 amino acids of beta-amylase 1 and 22 of beta-amylase 3 were dissimilar. The mRNA as well as protein sequences of *beta-amylase 1* and *beta-amylase 3* showed those to be of different origins.

Expression of sucrose biosynthesis genes

The expression of two genes of sucrose biosynthetic pathway, *UGPase* and *SPS2*, was studied in LT stressed and control leaves. Under LT, *UGPase* expression enhanced in the cold-tolerant as well as the cold-sensitive genotypes of chickpea (relative expression in ICC 16349: control = 0.89, LT = 1.36; relative expression in GPF2: control = 1.22, LT = 1.515) (figure 3a). The fold change relative to control was

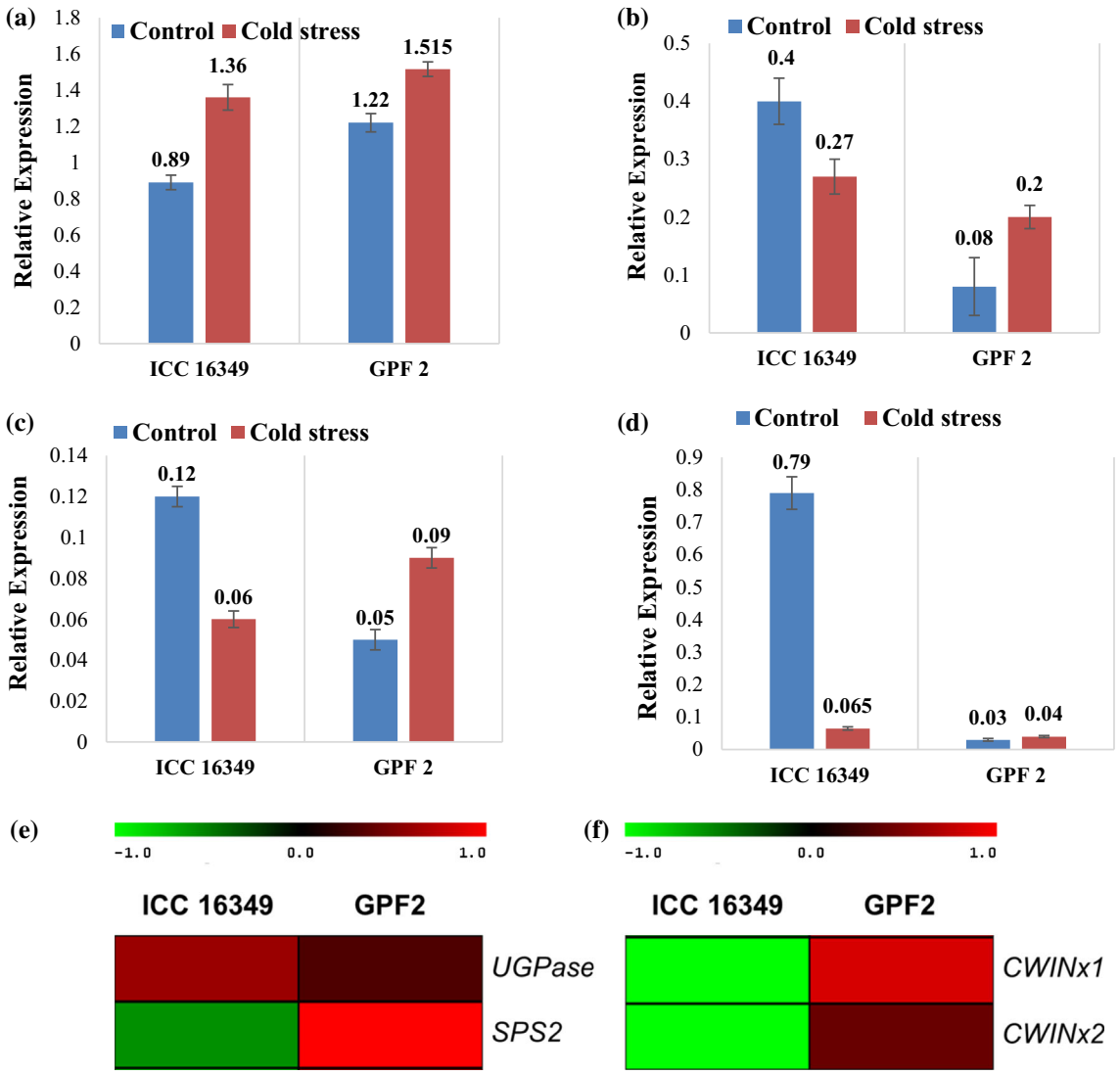

Figure 3. Relative expression (a, b, c, d) and heat maps (e, f) showing differential expression of sucrose metabolism genes in response to low temperature (4°C) for 72 h in leaves of chickpea genotypes ICC 16349 (cold-tolerant) and GPF2 (cold-sensitive). (a) *UGPase* (b) *SPS2* (c) *CWINx1* and (d) *CWINx2*. Temperature regime of 22°C / 16°C (day/night) acted as control.

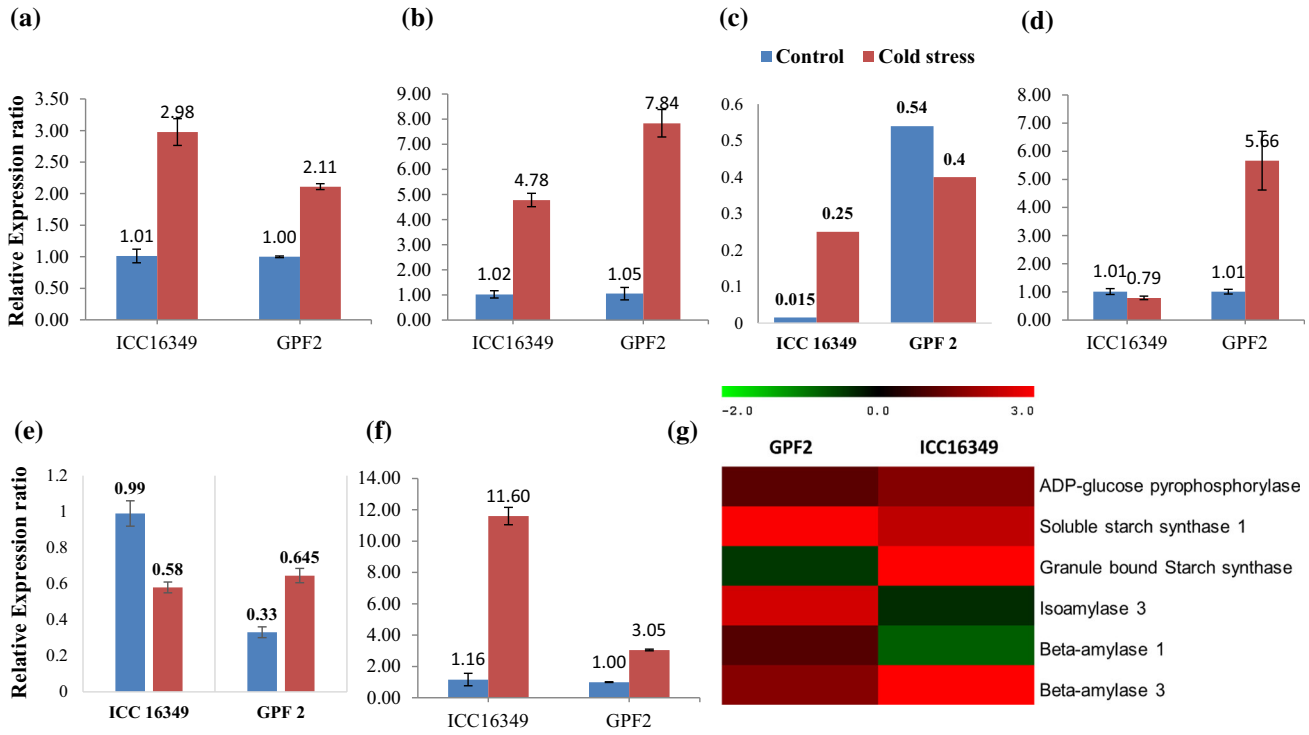

Figure 4. Relative expression (a, b, c, d, e, f) and heat map (g) showing differential expression of starch metabolism genes in response to low temperature (4°C) for 72 h in leaves of chickpea genotypes ICC 16349 (cold-tolerant) and GPF2 (cold-sensitive). (a) ADP-glucose pyrophosphorylase (b) soluble starch synthase 1 (c) granule-bound starch synthase 2, (d) isoamylase 3 (e) beta-amylase 1 and (f) beta-amylase 3. Temperature regime of 22°C / 16°C (day/night) acted as control.

higher in ICC 16349 (1.52-fold) than that in GPF2 (1.24-fold) (figure 3e) suggesting enhancement in UDP glucose accumulation in both the genotypes but more so in ICC 16349 than GPF2.

In contrast to *UGPase*, *SPS2* under cold stress downregulated in ICC 16349 (–1.48-fold, relative expression under control = 0.4, under LT = 0.27) but significantly upregulated in GPF2 (2.62-fold, relative expression: control= 0.08; LT = 0.21) (figure 3, b&e). The UGPase catalyzes synthesis of UDP glucose which is subsequently acted upon by SPS2 leading to the formation of sucrose-6-phosphate. The expression data pointed at accumulation of UDP glucose in the cold-tolerant ICC 16349 and that of sucrose-6-phosphate in cold sensitive GPF2.

Expression of sucrose transport and catabolism genes

Two paralogues of cell wall invertase, i.e. *CWINx1* and *CWINx2* were studied in the present study. Cell wall invertases are sucrose transporters that also catabolise conversion of sucrose molecules to simple sugars (Tang *et al*. 1999). Of the two paralogues, the expression of *CWINx1* was lower than *CWINx2* in both the genotypes, be it control or LT (figure 3, c&d). Under LT, these genes downregulated in ICC 16349 but upregulated in GPF2 (figure 3, c&d). In addition, the expression of *CWINx2* under control was considerably higher in ICC 16349 than in GPF2 (figure 3, c&d). The expression of *CWINx2* in LT treated ICC 16349 decreased by 12.15-fold (relative expression: LT, 0.06; control, 0.79) as compared to the control (figure 3, d&e). The expression of *CWINx2* though, very low in GPF2, both under control and cold stress (relative expression: control, 0.03; cold stress, 0.04) (figure 3d) did not downregulate under LT. In comparison to *CWINx2*, the expression of *CWINx1* under LT, decreased by two-fold in ICC 16349 and increased by 1.8 fold in GPF2 (figure 3c). The decrease in expression of *CWINx1* and *CWINx2* in ICC 16349 under cold stress suggested decreased transport of sucrose in the tolerant genotype. Since, the leaves are the source tissues, several fold decrease in the expression of *CWIN* genes in ICC 16349 suggested increased/sustained levels of sucrose in this genotype under LT as transport and conversion of sucrose to hexoses was reduced considerably. Of the two genes, only *CWINx2* expressed in relatively higher amounts as compared to *CWINx1* (figure 3, c&d) revealing independent transcriptional regulation of two paralogues of *CWIN* in chickpea (figure 3).

Expression of starch biosynthesis genes

The impact of LT on expression of three starch biosynthesis genes, i.e. *AGPase* (enzyme catalyzes the reaction: ATP +

Glucose-1-phosphate ⇔ ADP-Glucose + inorganic pyrophosphate), *SSSI* (enzyme uses ADP-glucose for chain elongation via α-1,4-glycosidic linkages) and *GBSS2* (enzyme contributes for the synthesis of amylose) was studied. The expression of *AGPase* enhanced in the cold-tolerant (ICC16349) as well as cold-sensitive chickpea (GPF2) genotypes with slightly higher expression in cold-tolerant ICC 16349 (relative expression: 2.9, fold change: 1.6) than cold-sensitive GPF2 (relative expression: 2.1, fold change: 1.1) (figure 4, a&g). Similar to *AGPase*, *SSS1* expression increased in GPF2 (relative expression: 7.8, fold change: 2.9) as well as ICC 16349 (relative expression: 4.8, fold change: 2.3) (figure 4, b&g). In contrast to these two genes, the expression of *GBSS2* increased in ICC 16349 but decreased in GPF2 (figure 4, c&g). In untreated ICC 16349, relative expression of *GBSS2* was very low (0.02) and that under LT increased to 0.25 (figure 4, c&g) with a fold change of 16.66 (figure 4, c&g). In contrast to ICC 16349, relative expression of *GBSS2* was decreased by 1.35-fold in GPF2, however, transcript abundance (0.4) under LT in GPF2 was higher than that in ICC 16349 (0.25) (figure 4, c&g). The expression profiles of three key starch biosynthesis genes in two genotypes suggested increased starch accumulation in cold-tolerant ICC 16349 as compared to control whereas starch content either remained static or decreased in cold-sensitive GPF2 owing to decreased *GBSS2* expression.

Expression of starch catabolism genes

Three key starch degradation genes, *isoamylase 3* (the enzyme catalyzes hydrolysis of (1->6)-alpha-D-glucosidic branch linkages in amylopectin), *BAM1* and *BAM3* (beta-amylases acts on starch molecules leading to generation of maltose by hydrolyzing the α-1,4-glucan linkages) showed differential expression under LT in the cold-tolerant and cold-sensitive genotypes. Under LT, the *isoamylase 3* downregulated in ICC 16349 (relative expression, 0.8; fold change, 0.4) and upregulated in GPF2 (relative expression, 5.7; fold change, 2.5) (figure 4, d&g). Similarly, under LT, *BAM1* downregulated in ICC 16349 (control: relative expression = 0.99; LT: relative expression = 0.58, fold change = –1.70) and upregulated in GPF2 (relative expression: LT= 0.65, control= 0.33, fold change = 1.95) (figure 4, e&g). The *BAM 3* under LT overexpressed both in ICC 16349 and GPF2 but upregulation was significantly higher in ICC 16349 (relative expression, 11.6; fold change, 3.5) as compared to GPF2 (relative expression, 3.1; fold change, 1.6). Differences in the expression of *BAM1* and *BAM3* suggested independent regulation of these genes in chickpea and complexity of *BAM* gene expression governing host responses under LT. The expression of starch biosynthesis and catabolism genes under LT implied that the starch accumulated in cold-tolerant ICC 16349 owing to increased synthesis and decreased degradation. Contrary to this, starch depletion was more in cold-sensitive GPF2 as all the three starch catabolism genes overexpressed under LT. In addition, *BAM3* overexpression in ICC 16349 pointed to higher maltose synthesis and accumulation in this genotype as compared to cold-sensitive GPF2.

Discussion

Carbohydrate metabolsim is important for plant development and for tolerating environmental stresses. Chickpea plants readjust carbohydrate contents and related enzyme activities when exposed to LT stress and dynamics of these changes vary between cold-tolerant and cold-sensitive chickpea genotypes, e.g. contents of total sugars, reducing sugars, starch and activities of sucrose synthase, inveratase and β-amylase under LT were higher in cold-tolerant lines of chickpea compared to cold-sensitive ones (Kaur *et al*. 2009; Kumar *et al*. 2011). In GPF2 (cold-sensitive genotype used in the present study), LT stress decreased the starch, sucrose and soluble sugar content (Kaur *et al*. 2008). The results (present study) indicated that *UGPase*, *SPS2*, *CWINx2*, *GBSS2*, *BAM1* and *BAM3* played important roles in LT responses of both the genotypes and readjustment of expression of these genes governed LT-tolerance in ICC 16349 (cold-tolerant genotype). All the genes including two paralogues of *CWIN* (*CWINx1* and *CWINx2*) and two beta amylase genes (*BAM1* and *BAM3*) were functional in the leaves of chickpea. Usually variants of a gene, e.g. TAI vacuolar invertase or LIN7 cell-wall invertase in tomato, vary in gene length but produce same proteins (Slugina *et al*. 2017, 2018), however, the *CWINx2* as well as the deduced protein of this gene was truncated versions of *CWINx1* gene or protein (present study) suggesting that *CWINx2* evolved from *CWINx1*. Both the genes expressed independently in two genotypes under control as well as LT suggesting independent regulation of these two genes. In contrast to *CWIN*, the *BAM1* and *BAM3* seem to evolve independently as mRNAs showed no similarity and proteins limited similarity. The UTRs of genes as well as paralogues also vary (present study; Slugina *et al*. 2017, 2018). A maize gene *Incw*1 encoding cell-wall invertase transcribed into two mRNAs, short and long, and variation in length was due to variable 3′UTR and not in the ORF or 5′UTR (Cheng *et al*. 1999). The 3′UTR in this case acted as a sensor for carbon starvation in maize cell (Cheng *et al*. 1999). Binding of UTR-interacting proteins to 3′UTR or change in length of 3′UTR have also been documented to contribute to abiotic stress tolerance by altering gene expression (Park *et al*. 2013; Catalán *et al*. 2016). Among the genes used in the present study, 3′UTRs of all were relatively longer with the exception of *SPS2* where it was relatively shorter. In contrast to this, 5′UTRs of *BAM1* and *BAM3* were very short whereas that of *SPS2* was very long.

Differential expression of the genes under LT in cold-tolerant and cold-sensitive genotypes revealed differences in

the mechanisms for coping LT by ICC 16349 and GPF2. The LT-induced overexpression of *UGPase* in ICC 16349 as well as GPF2 is indicative of increased biosynthesis of uridine diphosphoglucose (UDP-glucose) in both the genotypes (present study). The decrease in *SPS2* and *cell wall invertase* expression in ICC 16349 under LT indicated increased accumulation of UDP-glucose and increased/sustained levels of sucrose due to reduced sucrose catabolism. In contrast to ICC 16349, *SPS2* and *cell wall invertase* overexpression in GPF2 pointed to increased sucrose anabolism as well as catabolism under cold-sensitivity. The decrease in sucrose content in GPF2 under LT (Kaur *et al*. 2008) may thus partially be attributed to increased/sustained *CWIN* expression (present study), however, some other invertases, sucrose tranporters or SWEET genes might also be involved as expression of both paralogues of *CWIN* under study was very low in GPF2 even under control to explain considerable reduction in sucrose content in GPF2 under LT. The data, thus, suggested that UDPG and *UGPase* are involved in cold tolerance of ICC 16349. LT is already known to increase expression of *UGPase* in *Arabidopsis thaliana* (Ciereszko *et al*. 2001) and in *Populous* (Meng *et al*. 2007). The storage of potato tubers under cold also increased *UGPase* expression and its activity (Spychalla *et al*. 1994; Borovkov *et al*. 1996; Gupta and Sowokinos 2003). Upregulation of this gene is believed to be a major player in 'cold-sweetening' (Sowokinos *et al*. 1997; Kleczkowski *et al*. 2004). *UGPase* also acts as a regulatory entity that helps the plants to readjust the homeostatic mechanism (Ciereszko *et al*. 2001). It was suggested that *UGPase* expression is mediated via a hexokinase-independent and ABA-insensitive pathway (Ciereszko *et al*. 2001; Meng *et al*. 2007). The *UGPase* catalysed product, UDP-glucose, in addition to sucrose and free sugar formation, also biosynthesize cellulose, callose and hemicelluloses (Winter and Huber 2000; Meng *et al*. 2007; Führing *et al.* 2013). The gene *SPS* is known to be induced/upregulated by LT in *A. thaliana* (Usadel *et al*. 2008) as well as in several other crops (Guy *et al*. 1992; Hill *et al*. 1996; Reimholz *et al*. 1997; Sasaki *et al*. 2001; Essmann *et al*. 2008). Sucrose is a free sugar disaccharide, a well-known osmoprotectant (Levitt 1980), and a major transport form of carbohydrates in the majority of plants (Reimholz *et al*. 1997; Xu *et al*. 2017, 2019). The present study also revealed the mechanism by which cold-tolerant ICC 16349 maintained optimum sucrose levels under LT despite increasing UDPG synthesis. Under LT, ICC 16349 did not enhance sucrose synthesis but lowered its catabolism by lowering *CWIN* expression. In addition to leaves, cold stress-induced sucrose accumulation and expression of cell wall invertase genes have also been observed in pollen of rice and wheat (Oliver *et al*. 2005; Parish *et al*. 2012) and anthers of chickpea (Kiran *et al*. 2021).

Increased starch biosynthesis under abiotic stresses acts as a source sink and inhibits accumulation of free sugars that prevent or lower photosynthesis (Rosa *et al*. 2009; Dong and Beckles 2019). Starch is the end product of photosynthesis and its higher content in stressed plants signifies the stability of this process under stress (Kumar *et al*. 2011). Increased accumulation of starch under LT stress is a feature of cold-tolerant genotypes (ICC 16349 and ICC 16348) of chickpea but not of cold-sensitive genotypes (GPF2 and PBG 1) (Kaur *et al*. 2009; Kumar *et al*. 2011). Several fold increase in expression of *GBBS2* (starch synthesis enzyme) and decrease in expression of *isoamylase 3* and *BAM1* (enzyme for starch degradation) appears to be responsible for increased starch accumulation in the LT tolerant genotype ICC 16349 (present study). On the other hand, decline in *GBSS2* expression and several fold increase in expression of all three starch degradation enzymes explained reduced starch levels under LT in cold-sensitive GPF2 as observed earlier by Kaur *et al*. (2009) and Kumar *et al*. (2011). Similar to chickpea, higher expression of *GBSSI* gene in rice (*Oryza sativa* L.) leaves was observed at LT (15/10°C) than at 30/25°C (Wang *et al*. 2006). Rice grains increase amylose content under cold conditions vis-à-vis normal temperature (Asaoka *et al*. 1985; Hirano and Sano 1998) and this increased accumulation was attributed to an increase in GBSSI activity at LT (Suzuki *et al*. 2002). Starch breakdown is the primary function of BAM in plants (Kossmann and Lloyd 2000; Kaplan *et al*. 2006) and *BAM1* is induced by both cold and heat stress (Seki *et al*. 2001; Sung 2001). In *Arabidopsis*, 14-fold overexpression of *BAM* occurred as early as 2 h of exposure to cold stress (4°C) (Seki *et al*. 2001; Sung 2001). Similarly, in potato tubers, storage temperature reduction from 20°C to 5°C or 3°C, increased BAM activity by 4 to 5-fold after 10 days followed by maltose accumulation (Nielsen *et al*. 1997). Beta-amylase-induced cold tolerance is believed to be mediated by accumulation of maltose upon freezing stress wherein maltose contributed to the protection of membranes (Edner *et al*. 2007; Koide *et al*. 2011). Maltose under freezing stress also contributed to the protection of the photosynthetic electron transport chain (Edner *et al*. 2007). Significantly, higher *BAM3* expression in cold-tolerant ICC 16349 as compared to GPF2 pointed to the possible involvement of maltose in cold-tolerance in chickpea (present study).

In conclusion, the sucrose and starch metabolism genes retrieved from chickpea genome databases were functional in chickpea. *CWINx2* mRNA and its protein were the truncated versions of *CWINx1* suggesting possible evolution of *CWINx1* from *CWINx2*. In contrast to this, *BAM1* and *BAM3*, appeared to evolve independently. Both pairs of the genes, *CWINx1* and *CWINx2*, as well as *BAM1* and *BAM3* were functional with independent regulation as evident from differential expression of those in the cold-tolerant and cold-sensitive genotypes under LT and control. Cold-tolerance in ICC 16349 was governed by upregulation of *UGPase*, *GBSS2* and *BAM3*, and downregulation of *SPS2*, *CWINx1*, *CWINx2* and *BAM1*. Cold-sensitivity, on the other hand, was governed by lowered expression of *GBSS2* and increased expression of *SPS2*, *CWINx1*, *CWINx2* and *BAM1*. The gene expression data (present study) and sucrose starch contents

in ICC 16349 and GPF2 under LT (Kaur *et al*. 2009; Kumar *et al*. 2011) suggested that UDP-glucose, sucrose, starch, maltose, *UGPase*, *CWINs*, *GBSS2*, *isoamylase 3* and *BAM* are the important components of cold-tolerance machinery of chickpea.

Acknowledgements

The authors acknowledge financial support received from the Department of Biotechnology, GOI, New Delhi (grant number: BT/PR12371/AGR/2/901/2015).

References

Asaoka M., Okuno K. and Fuwa H. 1985 Effect of environmental temperature at the milky stage on amylose content and fine structure of amylopectin of waxy and nonwaxy endosperm starches of rice (*Oryza sativa* L.). *Agric. Biol. Chem.* **49**, 373–379.

Bhandari K., Sharma K. D., Rao B. H., Siddique K. H., Gaur P., Agrawal S. K. *et al*. 2017 Temperature sensitivity of food legumes: a physiological insight. *Acta Physiol. Plant.* **39**, 68.

Borovkov A. Y., Mcclean P. E., Sowokinos J. R., Ruud S. H. and Secor G. A. 1996 Effect of expression of UDP-glucose pyrophosphorylase ribozyme and antisense RNAs on the enzyme activity and carbohydrate composition of field-grown transgenic potato plants. *J. Plant Physiol.* **147**, 644–652.

Catalán A., Glaser-Schmitt A., Argyridou E., Duchen P. and Parsch J. 2016 An indel polymorphism in the MtnA 3′untranslated region is associated with gene expression variation and local adaptation in *Drosophila melanogaster*. *PLoS Genet.* **12**, e1005987.

Cheng W. H., Earl W. T. and Prem S. C. 1999 Sugars modulate an unusual mode of control of the cell-wall invertase gene (Incw1) through its 3′ untranslated region in a cell suspension culture of maize. *Proc. Natl. Acad. Sci. USA* **96**, 10512–10517.

Chinnusamy V., Zhu J. and Zhu J. K. 2007 Cold stress regulation of gene expression in plants. *Trends Plant Sci.* **12**, 444–451.

Ciereszko I., Johansson H. and Kleczkowski L. A. 2001 Sucrose and light regulation of a cold-inducible UDP-glucose pyrophosphorylase gene via a hexokinase-independent and abscisic acid-insensitive pathway in Arabidopsis. *Biochem. J.* **354**, 67–72.

Dèjardin A., Sokolov L. N. and Kleczkowski L. A. 1999 Sugar/osmoticum levels modulate differential abscisic acid-independent expression of two stress-responsive sucrose synthase genes in Arabidopsis. *Biochem. J.* **344**, 503–509.

Deryabin A. N., Dubinina I. M., Burakhanova E. A., Astakhova N. V., Sabel'nikova E. P. and Trunova T. I. 2005 Influence of yeast-derived invertase gene expression in potato plants on membrane lipid peroxidation at low temperature. *J. Therm. Biol.* **30**, 73–77.

Dinari A., Niazi A., Afsharifar A. R. and Ramezani A. 2013 Identification of upregulated genes under cold stress in cold-tolerant chickpea using the cDNA-AFLP approach. *PLoS One* **8**, 1–7.

Dong S. and Beckles D. M. 2019 Dynamic changes in the starch-sugar interconversion within plant source and sink tissues promote a better abiotic stress response. *J. Plant Physiol.* **234**, 80–93.

Edner C., Li J., Albrecht T., Mahlow S., Hejazi M., Hussain H., Kaplan F. *et al*. 2007 Glucan, water dikinase activity stimulates breakdown of starch granules by plastidial beta-amylases. *Plant Physiol.* **145**, 17–28.

Essmann J., Schmitz-Thom I., Schön H., Sonnewald S., Weis E. and Scharte J. 2008 RNA interference-mediated repression of cell wall invertase impairs defense in source leaves of tobacco. *Plant Physiol.* **147**, 1288–1299.

Führing J., Cramer J. T., Routier F. H., Lamerz A. C., Baruch P., Gerardy-Schahn R. and Fedorov R. 2013 Catalytic mechanism and allosteric regulation of UDP-glucose pyrophosphorylase from Leishmania major. *ACS Catal.* **3**, 2976–2985.

Garg R., Bhattacharjee A. and Jain M. 2015 Genome-scale transcriptomic insights into molecular aspects of abiotic stress responses in chickpea. *Plant Mol. Biol. Rep.* **33**, 388–400.

Gaur P. M., Krishnamurthy L. and Kashiwagi J. 2008 Improving drought-avoidance root traits in chickpea (*Cicer arietinum* L.)-current status of research at ICRISAT. *Plant Prod. Sci.* **11**, 3–11.

Gupta S. K. and Sowokinos J. R. 2003 Physicochemical and kinetic properties of unique isozymes of UDP-Glc pyrophosphorylase that are associated with resistance to sweetening in cold-stored potato tubers. *J. Plant Physiol.* **160**, 589–600.

Guy C. L., Huber J. L. and Huber S. C. 1992 Sucrose phosphate synthase and sucrose accumulation at low temperature. *Plant Physiol.* **100**, 502–508.

Hill L. M., Reimholz R., Schröder R., Nielsen T. H. and Stitt M. 1996 The onset of sucrose accumulation in cold-stored potato tubers is caused by an increased rate of sucrose synthesis and coincides with low levels of hexose-phosphates, an activation of sucrose phosphate synthase and the appearance of a new form of amylase. *Plant Cell Environ.* **19**, 1223–1237.

Hirano H. Y. and Sano Y. 1998 Enhancement of Wx gene expression and the accumulation of amylose in response to cool temperatures during seed development in rice. *Plant Cell Physiol.* **39**, 807–812.

Jain M., Misra G., Patel R. K., Priya P., Jhanwar S., Khan A. W. *et al*. 2013 A draft genome sequence of the pulse crop chickpea (*Cicer arietinum* L.). *Plant J.* **74**, 715–729.

Jha U. C., Chaturvedi S. K., Bohra A., Basu P. S., Khan M. S. and Barh D. 2014 Abiotic stresses, constraints and improvement strategies in chickpea. *Plant Breed.* **133**, 163–178.

Jung S. H., Lee J. Y. and Lee D. H. 2003 Use of SAGE technology to reveal changes in gene expression in Arabidopsis leaves undergoing cold stress. *Plant Mol. Biol.* **52**, 553–567.

Kohli D., Joshi G., Deokar A. A., Bhardwaj A. R., Agarwal M., Katiyar-Agarwal S. *et al*. 2014 Identification and characterization of wilt and salt stress-responsive microRNAs in chickpea through high-throughput sequencing. *PLoS One* **9**, e108851.

Koide T., Ohnishi Y. and Horinouchi S. 2011 Characterization of recombinant beta-amylases from *Oryza sativa*. *Biosci. Biotechnol. Biochem.* **75**, 793–796.

Kaloki P., Devasirvatham V. and Tan D. K. 2019 Chickpea Abiotic Stresses: combating drought, heat and cold. *In Abiotic and biotic stress in plants*. IntechOpen (https://doi.org/10.5772/intechopen.83404).

Kaplan F. and Guy C. L. 2004 β-Amylase induction and the protective role of maltose during temperature shock. *Plant Physiol.* **135**, 1674–1684.

Kaplan F., Sung D. Y. and Guy C. L. 2006 Roles of β-amylase and starch breakdown during temperatures stress. *Physiol. Plant.* **126**, 120–128.

Kaur G., Kumar S., Nayyar H. and Upadhyaya H. D. 2008 Cold stress injury during the pod-filling phase in chickpea (*Cicer arietinum* L.): Effects on quantitative and qualitative components of seeds. *J. Agron. Crop Sci.* **194**, 457–464.

Kaur S., Gupta A. K., Kaur N., Sandhu J. S. and Gupta S. K. 2009 Antioxidative enzymes and sucrose synthase contribute to cold stress tolerance in chickpea. *J. Agron. Crop Sci.* **195**, 393–397.

Kazemi-Shahandashti S. S., Maali-Amiri R., Zeinali H., Khazaei M., Talei A. and Ramezanpour S. S. 2014 Effect of short-term cold stress on oxidative damage and transcript accumulation of

defense-related genes in chickpea seedlings. *J. Plant Physiol.* **171**, 1106–1116.
Kiran A., Kumar S., Nayyar H. and Sharma K. D. 2019 Low temperature-induced aberrations in male and female reproductive organ development cause flower abortion in chickpea. *Plant Cell Environ.* **42**, 2075–2089.
Kiran A., Sharma P. N., Awasthi R., Nayyar H., Seth R., Chandel S. S., Siddique K. H., Zinta G. and Sharma K. D. 2021 Disruption of carbohydrate and proline metabolism in anthers under low temperature causes pollen sterility in chickpea. *Environ. Exp. Bot.*, https://doi.org/10.1016/j.envexpbot.2021.104500.
Kleczkowski L. A., Geisler M., Ciereszko I. and Johansson H. 2004 UDP-glucose pyrophosphorylase. An old protein with new tricks. *Plant Physiol.* **134**, 912–918.
Klotke J., Kopka J., Gatzke N. and Heyer A. G. 2004 Impact of soluble sugar concentrations on the acquisition of freezing tolerance in accessions of *Arabidopsis thaliana* with contrasting cold adaptation–evidence for a role of raffinose in cold acclimation. *Plant Cell Environ.* **27**, 1395–1404.
Kossmann J. and Lloyd J. 2000 Understanding and influencing starch biochemistry. *Crit. Rev. Plant Sci.* **19**, 171–226.
Kumar S., Nayyar H., Bhanwara R. K. and Upadhyaya H. D. 2010 Chilling stress effects on reproductive biology of chickpea. *J. SAT Agric. Res.* **8**, 1–14.
Kumar S., Malik J., Thakur P., Kaistha S., Sharma K. D., Upadhyaya H. D. *et al*. 2011 Growth and metabolic responses of contrasting chickpea (*Cicer arietinum* L.) genotypes to chilling stress at reproductive phase. *Acta Physiol. Plant.* **33**, 779–787.
Kumar S., Thakur P., Kaushal N., Malik J. A., Gaur P. and Nayyar H. 2013 Effect of varying high temperatures during reproductive growth on reproductive function, oxidative stress and seed yield in chickpea genotypes differing in heat sensitivity. *Arch. Agron. Soil Sci.* **59**, 823–843.
Liu M., Shi J. and Lu C. 2013 Identification of stress-responsive genes in *Ammopiptanthus mongolicus* using ESTs generated from cold-and drought-stressed seedlings. *BMC Plant Biol.* **13**, 88.
Levitt J. 1980 Responses of Plants to Environmental Stresses. In *Water, radiation, salt and other stress*, pp. 93–128. Field Crops Research, Academic Press, New York.
Mantri N. L., Ford R., Coram T. E. and Pang E. C. 2010 Evidence of unique and shared responses to major biotic and abiotic stresses in chickpea. *Environ. Exp. Bot.* **69**, 286–292.
Meng M., Geisler M., Johansson H., Mellerowicz E. J., Karpinski S. and Kleczkowski L. A. 2007 Differential tissue/organ-dependent expression of two sucrose-and cold-responsive genes for UDP-glucose pyrophosphorylase in Populus. *Gene.* **389**, 186–195.
Mo Y., Liang G., Shi W. and Xie J. 2011 Metabolic responses of alfalfa (*Medicago sativa* L.) leaves to low and high temperature induced stresses. *Afr. J. Biotechnol.* **10**, 1117–1124.
Muehlbauer F. J. and Rajesh P. N. 2008 Chickpea, a common source of protein and starch in the semi-arid ropics. *In Genomics of tropical crop plants*, pp. 171-186. Springer, New York.
Nägele T. and Heyer A. G. 2013 Approximating subcellular organisation of carbohydrate metabolism during cold acclimation in different natural accessions of *Arabidopsis thaliana*. *New Phytol.* **198**, 777–787.
Nielsen T. H., Deiting U. and Stitt M. 1997 A [beta]-amylase in potato tubers is induced by storage at low temperature. *Plant Physiol.* **113**, 503–510.
Oliver S. N., Van Dongen J. T., Alfred S. C., Mamun E. A., Zhao X., Saini H. S. *et al*. 2005 Cold-induced repression of the rice anther specific cell wall invertase gene *OSINV4* is correlated with sucrose accumulation and pollen sterility. *Plant Cell Environ.* **28**, 1534–1551.
Oufir M., Legay S., Nicot N., Van Moer K., Hoffmann L., Renaut J. *et al*. 2008 Gene expression in potato during cold exposure: changes in carbohydrate and polyamine metabolisms. *Plant Sci.* **175**, 839–852.
Parish R. W., Phan H. A., Iacuone S. and Li S. F. 2012 Tapetal development and abiotic stress: a centre of vulnerability. *Funct. Plant Biol.* **39**, 553–559.
Park S. H., Jeong J. S., Redillas M. C., Jung H., Bang S. W., Kim Y. S. and Kim J. K. 2013 Transgenic overexpression of UIP1, an interactor of the 3′ untranslated region of the Rubisco small subunit mRNA, increases rice tolerance to drought. *Plant Biotechnol. Rep.* **7**, 83–90.
Rani A., Devi P., Jha U. C., Sharma K. D., Siddique K. H. M. and Nayyar H. 2019 Developing climate-resilient chickpea involving physiological and molecular approaches with a focus on temperature and drought stresses. *Front. Plant Sci.* **10**, 1759.
Reimholz R., Geiger M., Haake V., Deiting U., Krause K. P., Sonnewald U. and Stitt M. 1997 Potato plants contain multiple forms of sucrose phosphate synthase, which differ in their tissue distributions, their levels during development, and their responses to low temperature. *Plant Cell Environ.* **20**, 291–305.
Rosa M., Prado C., Podazza G., Interdonato R., González J. A., Hilal M. and Prado F. E. 2009 Soluble sugars: Metabolism, sensing and abiotic stress: A complex network in the life of plants. *Plant Signal. Behav.* **4**, 388–393.
Ruelland E., Cantrel C., Gawer M., Kader J. C. and Zachowski A. 2002 Activation of phospholipases C and D is an early response to a cold exposure in Arabidopsis suspension cells. *Plant Physiol.* **130**, 999–1007.
Sasaki H., Ichimura K., Imada S. and Yamaki S. 2001 Sucrose synthase and sucrose phosphate synthase, but not acid invertase, are regulated by cold acclimation and deacclimation in cabbage seedlings. *J. Plant Physiol.* **158**, 847–852.
Savitch L. V., Gray G. R. and Huner N. P. 1997 Feedback-limited photosynthesis and regulation of sucrose-starch accumulation during cold acclimation and low-temperature stress in a spring and winter wheat. *Planta* **201**, 18–26.
Savitch L. V., Harney T. and Huner N. P. 2000 Sucrose metabolism in spring and winter wheat in response to high irradiance, cold stress and cold acclimation. *Physiol. Plant.* **108**, 270–278.
Seki M., Narusaka M., Abe H., Kasuga M., Yamaguchi-Shinozaki K., Carninci P. *et al*. 2001 Monitoring the expression pattern of 1300 Arabidopsis genes under drought and cold stresses by using a full-length cDNA microarray. *Plant Cell* **13**, 61–72.
Sharma K. K. and Lavanya M. 2002 Recent developments in transgenics for abiotic stress in legumes of the semi-arid tropics. JIRCAS Working Report No. 23. **23**, 61–73.
Sharma K. D. and Nayyar H. 2014 Cold stress alters transcription in meiotic anthers of cold tolerant chickpea (*Cicer arietinum* L.). *BMC Res. Notes.* **7**, 717.
Sharma K. D. and Nayyar H. 2016 Regulatory networks in pollen development under cold stress. *Front. Plant Sci.* **7**, 402.
Slugina M. A., Shchennikova A. V. and Kochieva E. Z. 2017 TAI vacuolar invertase orthologs: the interspecific variability in tomato plants (*Solanum* section Lycopersicon). *Mol. Genet. Genom.* **292**, 1123–1138.
Slugina M. A., Shchennikova A. V. and Kochieva E. Z. 2018 LIN7 cell-wall invertase orthologs in cultivated and wild tomatoes (*Solanum* section Lycopersicon). *Plant Mol. Biol. Rep.* **36**, 195–209.
Sofi S. A., Singh J., Chhikara N. and Panghal A. 2020 Effect of incorporation of germinated flour and protein isolate from chickpea on different quality characteristics of rice-based noodle. *Cereal Chem.* **97**, 85–94.
Sowokinos J. R., Thomas C. and Burrell M. M. 1997 Pyrophosphorylases in Potato (V. Allelic Polymorphism of UDP-Glucose Pyrophosphorylase in Potato Cultivars and Its Association with

Tuber Resistance to Sweetening in the Cold). *Plant Physiol.* **113**, 511–517.

Spychalla J. P., Scheffler B. E., Sowokinos J. R. and Bevan M. W. 1994 Cloning, antisense RNA inhibition, and the coordinated expression of UDP-glucose pyrophosphorylase with starch biosynthetic genes in potato tubers. *J. Plant Physiol.* **144**, 444–453.

Strand Å., Hurry V., Gustafsson P. and Gardeström P. 1997 Development of *Arabidopsis thaliana* leaves at low temperatures releases the suppression of photosynthesis and photosynthetic gene expression despite the accumulation of soluble carbohydrates. *Plant J.* **3**, 605–614.

Strand Å., Foyer C. H., Gustafsson P., Gardeström P. and Hurry V. 2003 Altering flux through the sucrose biosynthesis pathway in transgenic *Arabidopsis thaliana* modifies photosynthetic acclimation at low temperatures and the development of freezing tolerance. *Plant Cell Environ.* **26**, 523–535.

Sung D. Y. 2001 Characterization of Arabidopsis heat shock protein 70 (Hsp70) gene family and microarray analysis of gene expression in response to temperature extremes. Ph.D. dissertation, University of Florida, Florida.

Suzuki Y., Sano Y. and Hirano H. Y. 2002 Isolation and characterization of a rice mutant insensitive to cool temperatures on amylose synthesis. *Euphytica* **123**, 95–100.

Tang G. Q., Luscher M. and Sturm A. 1999 Antisense repression of vacuolar and cell wall invertase in transgenic carrot alters early plant development and sucrose partitioning. *Plant Cell* **11**, 177–189.

Thakur A., Sharma K. D., Siddique K. H. and Nayyar H. 2020 Cold priming the chickpea seeds imparts reproductive cold tolerance by reprogramming the turnover of carbohydrates, osmo-protectants and redox components in leaves. *Sci. Horti.* **261**, 108929.

Tesfaye K., Walker S. and Tsubo M. 2006 Radiation interception and radiation use efficiency of three grain legumes under water deficit conditions in a semi-arid environment. *Eur. J. Agron.* **25**, 60–70.

Usadel B., Bläsing O. E., Gibon Y., Retzlaff K., Höhne M., Günther M. and Stitt M. 2008 Global transcript levels respond to small changes of the carbon status during progressive exhaustion of carbohydrates in Arabidopsis rosettes. *Plant Physiol.* **146**, 1834–1861.

Varol I. S., Kardes Y. M., Irik H. A., Kirnak H. and Kaplan M. 2020 Supplementary irrigations at different physiological growth stages of chickpea (*Cicer arietinum* L.) change grain nutritional composition. *Food Chem.* **303**, 125402.

Varshney R. K., Song C., Saxena R. K., Azam S., Yu S., Sharpe A. G. *et al*. 2013 Draft genome sequence of chickpea (*Cicer arietinum*) provides a resource for trait improvement. *Nat. Biotechnol.* **31**, 24–26.

Wang S. J., Liu L. F., Chen C. K. and Chen L. W. 2006 Regulations of granule-bound starch synthase I gene expression in rice leaves by temperature and drought stress. *Biol. Plant.* **50**, 537–541.

Wang K., Shao X., Gong Y., Zhu Y., Wang H., Zhang X. *et al*. 2013 The metabolism of soluble carbohydrates related to chilling injury in peach fruit exposed to cold stress. *Postharvest Boil. Technol.* **86**, 53–61.

Winter H. and Huber S. C. 2000 Regulation of sucrose metabolism in higher plants: localization and regulation of activity of key enzymes. *Crit. Rev. Plant Sci.* **19**, 31–67.

Xu X. X., Hu Q., Yang W. N. and Jin Y. 2017 The roles of cell wall invertase inhibitor in regulating chilling tolerance in tomato. *BMC Plant Biol.* **17**, 1–3.

Xu F., Wang K., Yuan W., Xu W., Liu S., Kronzucker H. J. *et al*. 2019 Overexpression of rice aquaporin OsPIP1; 2 improves yield by enhancing mesophyll CO2 conductance and phloem sucrose transport. *J. Exp. Bot.* **70**, 671–681.

Yamaguchi-Shinozaki K. and Shinozaki K. 2006 Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annu. Rev. Plant Biol.* **57**, 781–803.

Yue C., Cao H. L., Wang L., Zhou Y. H., Huang Y. T., Hao X. Y. *et al*. 2015 Effects of cold acclimation on sugar metabolism and sugar-related gene expression in tea plant during the winter season. *Plant Mol. Biol.* **88**, 591–608.

Corresponding editor: Manoj Prasad