RESEARCH ARTICLE

Integrated approaches to study the drought tolerance mechanism in hot pepper (*Capsicum annuum* L.)

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Abstract Drought is one of the predominant abiotic stresses which have phenomenal impact on crop productivity. Alterations in aquaporin gene expressions are part of complex molecular responses by plant in response to drought. To better understand the role of aquaporins in economically important crop chilli (*Capsicum annuum*), drought induced gene expression of twelve aquaporins was determined in drought tolerant—KCa-4884 and drought susceptible—G-4 genotypes. Conjointly, the effect of drought on leaf water status and photosynthetic parameters were evaluated. Gene expression of all examined 12 aquaporins was up-regulated in KCa-4884 and in contrast, all the aquaporin genes were down-regulated in G-4 under drought stress. Significant variations among two chilli genotypes have been recorded in photosynthetic rate (P_n), stomatal conductance (G_s), and relative water content (RWC), sub-stomatal CO_2 concentration (C_i). KCa-4884 revealed significantly high rates of P_n and RWC and decreased G_s under water deficit conditions providing evidence for superior drought adaptive strategies. Differences in physiological parameters illustrate prevention of water loss during drought. Up-regulation of aquaporins in drought tolerant genotype implicates their possible role in

water relations and photosynthetic performance even under extended drought conditions.

Keywords Aquaporins · *Capsicum annuum* · Photosynthetic rate · Stomatal conductance · Relative water content · Up-regulation · Down-regulation

Introduction

Drought, one of the adverse environmental stresses, impairs the growth and development of plants. To counterattack such unfavourable growth conditions, plants have developed unique defence mechanisms and processes for acclimation that enhance their tolerance to unfavourable conditions. Drought accounts for the reduction in crop yield in as much as half of the world's irrigated land. Diverse physiological, molecular and cellular events occur under such stress to confer tolerance against drought (Zhu 2016). Even though a large number of genes induced by drought were identified, the biological roles of these genes with regard to either stress tolerance or sensitivity are largely ambiguous in higher plants. Therefore, studying the functions of stress inducible genes become indispensable to comprehend the molecular mechanism of stress tolerance in plants.

Water transport is known to be controlled by water channel proteins, aquaporins (AQPs) which belong to membrane intrinsic proteins (MIPs) family. In plants, AQP mediated water transport has been implicated to play a major role in varied biological processes such as seed germination (Liu et al. 2015), cell elongation (Obroucheva and Sinkevich 2010) and leaf hydraulic and stomatal conductance (Pou et al. 2013). To date, AQPs are divided into plasma membrane intrinsic proteins (PIPs), tonoplast

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intrinsic proteins (TIPs), nodulin-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs), unclassified X intrinsic proteins (XIPs), homologue of the *Escherichia coli* intrinsic protein GlpF (GIPs), hybrid intrinsic proteins (HIPs) (Anderberg et al. 2012). Usually, all green plants contain PIPs, TIPs, NIPs, and SIPs. XIPs are found in some dicots (Venkatesh et al. 2013) but were absent in monocots (Danielson and Johanson 2008). HIPs and GIPs were reported to be present in fern (*Selaginella moellendorffii*) and moss (*Physcomitrella patens*) (Kong et al. 2017).

One of the prerequisite and predominant responses of a plant cell to water stress is regulation of AQPs (Prado and Maurel 2013). Several studies indicate that AQPs are responsible to play crucial role in drought stress tolerance by precisely regulating water movement (Sutka et al. 2016). Transcriptional regulation of AQPs becomes a vital mechanism irrespective of other important regulatory mechanisms such as post-translational modifications, heteromerisation and gating (Li et al. 2014). As AQPs maintain water balance, drought induced AQP expression has been correlated with alleviation of drought in many crops. Drought induced up-regulation and down-regulation of AQPs (TIPs, PIPs, NIPs and SIPs) has been reported in *Phyllostachys edulis* (Sun et al. 2016). Drought induced dramatic changes in transcript abundance of PIPs, TIPs and SIPs has been observed in *Sorghum bicolor* (Reddy et al. 2015). Influence of AQP expression (PIPs and TIPs) on water status and physiological parameters under drought stress has been documented in *Robinia pseudoacacia* (He et al. 2016). Further correlation between AQP (PIPs) expressions and quantitative changes in stomatal conductance and photosynthesis and mesophyll conductance was documented (Perez-Martin et al. 2014). Augmented transcript levels of CaPIP1-1 during chilling stress, mannitol stress, salt stress, *Phytophthora capsici* infection in pepper was also reported (Yin et al. 2015).

Hot pepper (*Capsicum annuum*) is one of the widely cultivated crops that belong to family Solanaceae. It is an important commercial crop grown in India. India has emerged as leading producer and exporter of hot pepper contributing one fourth of world's production. Irrigation often becomes limiting factor as hot pepper cultivation is mainly restricted to warm and semiarid regions resulting in reduction in yield. Although there are several reports affirming role of AQP genes in various stresses, meagre information is known about drought induced differential expression of aquaporin genes in pepper cultivars. Hence, the present work aimed at understanding differential expression of drought induced AQPs in conjunction with physiological changes in chilli genotypes.

Materials and methods

Database search and identification

To identify and extract aquaporin proteins following NCBI accessions viz., XP_016557383.1, PHT59475.1, XP_016576910.1, PHT46496.1, PHU01844.1, PHT33304.1, PHU26047.1, PHT55846.1, PHT48666.1, XP_016573514.1, PHU18228.1, PHT48666.1, ADO66672.1, ADO66671.1, NP_001292491.1 were used to query the CM334 (v.155) *Capsicum annuum* genome database (<https://solgenomics.net/>) using BLASTP. After filtering *Capsicum annuum* AQPs (CaAQPs) with at least 70% identity with query sequence, AQP proteins were aligned to ensure that no protein was repeated. Remaining aquaporin proteins were examined for presence of domain (MIP) using pfam (<http://pfam.xfam.org/search/sequence>). Two NPA motifs, ar/R selectivity filter (H2, H5, LE1, LE2) and Froger's positions (P1–P5) were inferred from multiple sequence alignment with structure resolved *Spinacia oleracea* PIP2;1 (Hove and Bhavé 2011). Physicochemical properties such as length, molecular weight, Isoelectric point (pI), were obtained using PROTPARAM tool available on the Expert Protein Analysis System (ExPASy) proteomics server (<https://web.expasy.org/protparam/>). Further sub-cellular localization prediction of proteins was carried out using Cello v.2.5 sub-cellular localisation predictor (<http://cello.life.nctu.edu.tw>). Number of trans-membrane helices was deduced using HMMTOP v.2 (<http://www.enzim.hu/hmmtop/>). Also, the maximum, minimum and average percentage of identity amongst selected AQP genes and proteins were assessed using SIAS tool available on the Immunomedicine Group (<http://imed.med.ucm.es/Tools/>).

Plant material and growth conditions

Seeds of two pepper genotypes KCa-4884 (drought tolerant) and G-4 (drought susceptible) were procured from Horticulture Research Station, Lam Farm, Guntur, Andhra Pradesh. Seeds were sown in black trays containing a mixture of peat and vermiculite (2:1 v/v) for 45 days. Plants were grown in greenhouse under controlled conditions—16 h light/8 h dark photoperiod at 27 °C during the day and 21 °C at night, and watered regularly. After 45 days, seedlings were transplanted to pots and allowed for acclimatisation for one week. Both hot pepper genotypes were subjected to two water regimes viz., 100% Field Capacity (Control-C) and 40% Field Capacity (Stress-S) for one week. Gravimetric method was adapted for precise imposition of water stress (Ramegowda et al. 2013). Water stress was maintained at required Field Capacity by

weighing the pots twice a day and replenishing the water lost by evapotranspiration.

Gas exchange measurements

After one week of stress imposition, young and fully expanded third leaf from upper canopy was placed in the leaf chamber, allowed to adjust for 2–3 min to the new microclimate until the measurements were stabilized and Net Photosynthetic Rate (P_n ; $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), Stomatal conductance (G_s ; $\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) and sub-stomatal CO_2 concentration (C_i) were measured using a portable $\text{CO}_2/\text{H}_2\text{O}$ infra-red gas analyzer IRGA (ADC Bioscientific Ltd., UK). Gas exchange parameters were taken at saturating photosynthetically active radiation of $1600 \mu\text{mol (photon)} \text{ m}^{-2} \text{ s}^{-1}$ supplied by an LED light source (LC pro-32,070-Broad; ADC Bioscientific Ltd) attached to the leaf chamber, air temperature of $25\text{--}26^\circ\text{C}$, leaf temperature was 28°C , relative humidity of $55\text{--}60\%$ and CO_2 concentration $300 \mu\text{mol CO}_2 \text{ mol}^{-1}$ between 10.00 and 13.00 (Solar time).

Leaf water status

Leaf water status in two genotypes was determined by evaluating the relative water content (RWC). Leaf disks of 10 mm in diameter were excised from the interveinal areas of each plant. For each replicate, 30 discs were pooled and their fresh weights (FW) were determined. They were refloated on distilled water in petri dishes for 4 h to regain turgidity and then the turgid tissues were blotted to remove excess water and weighed for turgid weight (TW). The samples were dried at 80°C for 24 h to determined dry weight (DW). Leaf RWC was calculated according to Rouphael et al. (2008) using formula $\text{RWC} = [(\text{FW} - \text{DW})/(\text{TW} - \text{DW})] \times 100$, where FW is fresh weight of leaf discs, TW is the turgid weight after rehydration and DW is dry weight of leaf discs.

Primer design for aquaporin genes

Coding sequences (CDS) and gene sequences of deduced AQP protein sequences of pepper were retrieved from Sol Genomics Network (<https://solgenomics.net/>). RT-qPCR primer sets were designed in 3' and 5' untranslated regions of each gene to evade non-specific amplification of aquaporin genes (Bárcana et al. 2014a, b) using Prime Quest Tool (<http://eu.idtdna.com/PrimerQuest/Home/Index>). All the primer sequences of AQPs and reference gene (UBI-3) are shown in Table 1.

RNA extraction

Leaf samples for RNA extraction were immediately frozen in liquid nitrogen and stored at -80°C until analysis. RNA was extracted from leaf tissue from stressed and control plants of both genotypes according to acid guanidinium thiocyanate-phenol-chloroform extraction procedure (Chomczynski and Sacchi 2006). Leaf tissue (100 mg) was ground in liquid nitrogen to a fine powder, using a mortar and pestle, and then the powder was further homogenized using RNA Buffer. Contents were centrifuged for 10 min at 12,000 rpm. Supernatant was transferred to 2 ml Eppendorf tubes and equal aliquots of chloroform were added. Eppendorf tubes were subjected to centrifugation at 12,000 rpm for 10 min. Upper aqueous layer was transferred to 1.5 ml Eppendorf tubes. To this solution, ice cold propanol, 1.5 M NaCl was added and incubated for 5 min at 4°C . After incubation, solution was centrifuged at 12,000 rpm for 10 min and supernatant was discarded. Pellet was washed with ethanol and air dried. After complete drying, pellet was re-suspended into DEPC water. To remove residual genomic DNA, RNA samples were treated with DNase I (RNase-free) (Takara- cat # 2270B). Purity and integrity of RNA were estimated by calculating $\text{A}_{260}/\text{A}_{280}$ and $\text{A}_{260}/\text{A}_{230}$ absorbance ratios and agarose gel (1.5%) electrophoresis analysis respective.

RT-qPCR

cDNA was synthesized in triplicate from $3 \mu\text{g}$ of RNA using prime script 1st strand cDNA synthesis kit (Takara-cat # 6110A.). Gene expression was analyzed using reverse transcription quantitative PCR (RT-qPCR) (QuantStudio3-Applied Biosystems). RT-qPCR reaction was performed in a total volume of $12 \mu\text{l}$ (Sybr Green— $6 \mu\text{l}$, cDNA— $2 \mu\text{l}$, Forward Primer— $1 \mu\text{l}$, Reverse Primer— $1 \mu\text{l}$, DEPC water— $2 \mu\text{l}$) using MicroAmp Fast 96-Well Reaction Plates covered with MicroAmp Optical Adhesive Film (Applied Biosystems). Standard cycling parameters were used and raw data were normalized using UBI-3 reference gene. Baseline, thresholds were set manually and CT values were calculated by the software. Efficiency of RT-qPCR reaction for each aquaporin gene was calculated from the standard curve obtained by serial dilutions of pooled cDNA. Relative gene expression between the samples was calculated using $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen 2001). This method allows presenting an estimated level of gene expression based on the supposition of 100% PCR efficiency of target and reference genes. The RT-qPCR products obtained were checked on 2% agarose gel. The presence of single band with expected amplicon size was considered as specific amplification (Hachez et al. 2006).

Table 1 Primers used for aquaporin expression analysis

Gene Id [*]	AQP name	Subfamily	Forward primer	Reverse primer
CA01g24010	CaAQP-1	NIP7-1	GAACCCAGCCAGGTCTTTAG	CTCCTGCAACAGCTCCTATAAT
CA02g06180	CaAQP-2	NIP4-2 ^a	TGGATCAGTTGCGGTGAATAA	GATACGTGTCCCACAGAGTAAAT
CA06g22180	CaAQP-3	NIP1-1 ^a	CGTTGTCACACTACCTGGAAT	CAATGGTAACAGCAGGGTTAAAG
CA00g57100	CaAQP-4	NIP1-2	GCCAGTATCAGGAGCATCAA	GCCCAACTATGTAAACCCAAAG
CA02g13220	CaAQP-5	NIP1-3	GGAGAACTCACTGGACTTGTAT	CAAACCTCTTGC GGGATTTCATT
CA03g12700	CaAQP-6	PIP1-1 ^a	GGCCTGGACGCTGAAATAA	CAAGACTCCTAACTGGGTTGATAC
CA05g16220	CaAQP-7	PIP1-2	GGATTTGCGGTGTTCCTAGT	TGCGGGTTCCTTGTATAGATG
CA11g14820	CaAQP-8	TIP1-2 ^a	AACATCTTGTCTGGTGGTACTG	ACCCAATGGTTGGTCCATATC
CA06g12470	CaAQP-9	TIP1-3	TGAACCCAGCTCGATCATTT	CCATAAATAAACCCGGCCAATC
CA03g15670	CaAQP-10	TIP5-1	GGGATCTATGAACCCTGCTTAC	TGCAGGGAAGACCACATTATC
CA04g14660	CaAQP-11	XIP1-1	GGCCATGTCACGTATTGTCTA	GAAACACCTAGCCGGATTCA
CA08g18710	CaAQP-12	XIP1-2	GGCAGGCATTCTTGTGTTTATT	AGCACCTAGCTGGATTCATTC
Reference Gene	Ubiquitin	UBI-3	TGTCCATCTGCTCTCTGTTG	CACCCCAAGCACATAAGAC

^{*}Gene Id as present in Sol Genomics Network, ^aAQP Name as given in NCBI

Statistical analysis

All the data on photosynthetic gas exchange parameters, leaf water status was obtained from three biological replicates and represented as mean $\pm$ standard deviation. In gene expression studies, for all the four samples (KCa-4884 control, KCa-4884 stressed, G-4 control and G-4 stressed), three independent biological replicates, each with two technical replicates were maintained. Data were represented as mean $\pm$ standard deviation. All the physiological data and gene expression data were tested for significance between tolerant and susceptible genotypes using analysis of variance (ANOVA) at 5% probability. Heatmap to depict relative gene expression was generated using Heatmapper (<http://heatmapper.ca/>) (Babicki et al. 2016).

Results

Identification of *Capsicum annuum* aquaporins (CaAQPs)

Using fifteen aquaporin proteins as query sequences, 12 aquaporin proteins were extracted from pepper genome (CM334 v.1.55) (<https://solgenomics.net>). CaAQP protein sequences and CDS sequences are given in supplementary files- 1 and 2. Multiple Sequence alignments were analysed carefully and conserved amino acid residues and motifs were identified indicating that they are functional channels of water and other solutes. Sequence analysis showed that 12 CaAQPs ranged from 125 to 329 amino acids with a theoretical molecular weight of 13.77–35.28 kDa and pI

value ranging from 5.67 to 9.26. AQP characteristics such as number of amino acids (length), molecular weight (MW), isoelectric point (pI) were tabulated in Table 2. Sequence cluster analysis of 12 CaAQPs with known AQPs further divided them into four subfamilies—viz, NIPs, PIPs, TIPs and XIPs. Similarity analysis of 12 CaAQP proteins and CDS revealed a high sequence diversity existing within and among four families (Tables 3, 4).

Effect of drought on photosynthetic rate, stomatal conductance and sub-stomatal CO₂ concentration

Drought stress showed significant effect on stomatal conductance and photosynthetic rate. In control conditions, KCa-4884 exhibited high rates of G_s ($0.23 \text{ mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) (Fig. 1b) and P_n ($19.58 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) (Fig. 1a). Under stress, G_s and P_n reduced by 30% and 68.64% respectively in KCa-4884. At 40% FC, photosynthetic rate was significantly reduced by 73.80% in G-4 while there was only 16.66% reduction in stomatal conductance. Under controlled conditions, C_i was $228 \mu\text{mol CO}_2 \text{ mol}^{-1}$ and $265 \mu\text{mol CO}_2 \text{ mol}^{-1}$ in KCa-4884 and G-4 respectively (Fig. 1c). There was observed increase in C_i in both genotypes and increase in KCa-4884 was found to be 19.29% ($272 \mu\text{mol CO}_2 \text{ mol}^{-1}$) while the increment was 4.52% ($277 \mu\text{mol CO}_2 \text{ mol}^{-1}$) in G-4 (Fig. 1c).

Leaf water status

Drought adversely effected water status of the both the genotypes. At control conditions, KCa-4884 and G-4 maintained 87.71% and 80.16% RWC respectively (Fig. 1d). When the genotypes were subjected to water

Table 2 Structural and subcellular localization analysis of the CaAQPs

AQP name	Length	MW (kDa)	pI	TM	Location	Ar/R				NPA		Froger's positions				
						H2	H5	LE1	LE2	LB	LE	P1	P2	P3	P4	P5
CaAQP-1	211	21.99	9.26	5	Plasma membrane	V	L	S	R	NPA	NPA	A	S	A	Y	V
CaAQP-2	295	31.48	6.71	6	Plasma membrane	M	I	A	R	NPA	NPA	A	S	A	Y	I
CaAQP-3	290	31.63	9.02	6	Plasma membrane	M	F	S	R	NPA	NPA	V	S	A	Y	I
CaAQP-4	272	29.06	8.40	6	Plasma membrane	M	F	A	R	NPA	NPA	A	S	A	Y	I
CaAQP-5	246	26.44	9.25	6	Plasma membrane	M	A	A	R	NPA	NPA	A	S	A	Y	I
CaAQP-6	221	24.24	8.24	5	Plasma membrane	F	I	T	R	NLV	NPV	M	S	T	Y	F
CaAQP-7	286	30.73	9.10	6	Plasma membrane	F	I	T	R	NPA	NPA	M	S	A	F	W
CaAQP-8	125	13.77	6.02	3	Plasma membrane	–	I	G	M	–	NPA	–	S	A	Y	W
CaAQP-9	250	25.18	5.67	6	Plasma membrane	F	I	A	R	NPA	NPA	A	S	A	Y	W
CaAQP-10	256	26.69	8.95	6	Plasma membrane	S	G	T	Y	NPA	NPA	S	S	A	Y	W
CaAQP-11	326	34.86	7.08	7	Plasma membrane	T	I	S	R	NPV	NPA	A	C	A	F	W
CaAQP-12	329	35.28	7.62	7	Plasma membrane	T	F	S	R	SPV	NPA	A	C	A	F	W

MW molecular weight, pI isoelectric point, TM number of transmembrane helices

Table 3 Percentage of Similarity among 12 Capsicum annum AQP Proteins

Similarity (%)	CaAQP-1	CaAQP-2	CaAQP-11	CaAQP-20	CaAQP-44	CaAQP-4	CaAQP-8	CaAQP-30	CaAQP-38	CaAQP-41	CaAQP-40	CaAQP-50
CaAQP-1	100											
CaAQP-2	14.21%	100										
CaAQP-11	11.37%	21.80%	100									
CaAQP-20	14.69%	16.58%	17.06%	100								
CaAQP-44	10.90%	15.16%	18%	14.21%	100							
CaAQP-4	19.43%	13.74%	16.58%	12.79%	20.85%	100						
CaAQP-8	13.27%	18.48%	17.06%	18%	15.63%	16.58%	100					
CaAQP-30	9.95%	6.63%	9.95%	6.63%	8.05%	16.11%	7.58%	100				
CaAQP-38	17.06%	13.27%	15.16%	18%	21.32%	20.37%	15.16%	8.53%	100			
CaAQP-41	11.84%	16.11%	17.53%	17.53%	15.63%	13.27%	12.79%	5.68%	22.27%	100		
CaAQP-40	20.85%	23.22%	26.06%	22.27%	10.90%	16.11%	19.43%	9.95%	17.06%	15.16%	100	
CaAQP-50	17.53%	20.37%	19.90%	20.85%	17.53%	17.06%	18.95%	6.63%	18%	17.06%	26.06%	100

Minimum Similarity- 5.68

Maximum Similarity- 100

Mean- 22.83

SD- 23.684133

deficit conditions, RWC was reduced by 0.44 folds in KCa-4884 (48.40%), while in G-4 (18.33%), the reduction of RWC was by 0.77 folds (Fig. 1D).

Aquaporin family gene expression analysis

We observed significant variations in the expression profiles of AQPs at control and stress conditions in both KCa-4884 and G-4 genotypes (Fig. 2). Transcript abundance of all the aquaporins was increased in drought and decreased

in tolerant and susceptible genotypes respectively under drought stress (Fig. 3). In KCa-4884, CaAQP-8 was increased by 2.83 folds followed by CaAQP-11 (2.73 folds) and CaAQP-9 showed lowest increase (1.66 folds) under drought stress. In G-4, expression levels of CaAQP-1 and CaAQP-10 decreased by 3.76 folds and 2.86 folds respectively. Decrease in transcript abundance was very low in CaAQP-11 (0.86), followed by CaAQP-2 (1.25) (Fig. 3).

Table 4 Percentage of Similarity between CaAQP CDS sequences

Similarity (%)	CaAQP-1	CaAQP-2	CaAQP-11	CaAQP-20	CaAQP-44	CaAQP-4	CaAQP-8	CaAQP-30	CaAQP-38	CaAQP-41	CaAQP-40	CaAQP-50
CaAQP-1	100											
CaAQP-2	24.84%	100										
CaAQP-11	22.32%	38.99%	100									
CaAQP-20	25.94%	39.15%	38.99%	100								
CaAQP-44	29.55%	33.33%	33.17%	30.50%	100							
CaAQP-4	27.83%	29.08%	28.14%	30.03%	29.24%	100						
CaAQP-8	24.68%	34.59%	38.20%	37.10%	30.97%	28.14%	100					
CaAQP-30	16.03%	16.03%	17.13%	17.61%	15.40%	19.02%	16.50%	100				
CaAQP-38	29.71%	31.91%	31.60%	32.70%	33.33%	30.03%	32.07%	15.72%	100			
CaAQP-41	27.83%	31.60%	32.07%	34.27%	33.01%	29.71%	30.66%	15.09%	36.47%	100		
CaAQP-40	30.81%	41.03%	34.59%	38.67%	29.55%	29.71%	36.16%	16.82%	33.96%	35.84%	100	
CaAQP-50	26.10%	38.05%	36.16%	35.22%	30.50%	28.77%	35.69%	17.29%	33.17%	32.54%	41.03%	100
Minimum similarity- 15.09												
Maximum similarity- 100												
Mean- 35.5825												
SD- 20.5573												

Discussion

Plant susceptibility or tolerance to drought stress is dependent on amplitude of stress, duration of stress period, plant species, their development stage and interactions among other stress factors (Demirevska et al. 2009). Drought stress engenders distinctive and diverse plant responses that encompass several physiological, metabolic and molecular adjustments. Hence, we investigated drought induced physiological and aquaporin gene expressions in two hot pepper genotypes differing in drought tolerance, upon imposing severe water stress for one week at vegetative phase.

Imposition of drought stress resulted in the decrease of net photosynthesis in both drought tolerant and susceptible genotypes. Reddy et al. (2017) also observed significant decrease of P_n in mulberry genotypes upon drought imposition. This negative impact on photosynthesis is due to consequence of increased leaf resistance to CO_2 diffusion due to decreased leaf water potential (Pflug et al. 2018). Nevertheless, KCa-4884 maintained relatively higher assimilation power in comparison with G-4. KCa-4884 being tolerant was able to maintain relatively higher water status thereby reducing the damage caused by drought. Reduced photosynthesis is due to stomatal closure in drought tolerant species while, the same is due to damage to plant in drought sensitive species (Toscano et al. 2016).

When plants were subjected to severe drought, stomatal conductance has been reduced in KCa-4884 and G-4.

Drought induced decline in stomatal conductance has also been reported in maize seedlings (Chen et al. 2016), mulberry genotypes (Reddy et al. 2017) and wheat genotypes (Ouyang et al. 2017). At water deficit conditions, plants partially close stomata to prevent water loss. Though this can be attributed as adaptability to water stress, it also reduces photosynthesis due to decreased CO_2 influx as stomata regulate CO_2 diffusion into, and water diffusion out of, plant leaves (Pinheiro and Chaves 2010). This is manifested as reduced assimilation in KCa-4884 although it retained higher leaf water status.

Though drought stress did not decrease stomatal conductance drastically in G-4, magnitude of decrease in net photosynthetic rate was significant. This may indicate the lack of stomatal control over leaf gas exchange parameters. Under drought conditions, non-stomatal inhibition of photosynthesis also has a major influence. Also, increase of sub-stomata CO_2 concentration in both genotypes under drought conditions also signifies the predominance of non-stomatal limitations. Reduced photosynthesis under drought can be ascribed to both stomatal and non-stomatal (mesophyll resistance, impaired chloroplast function) factors (Mafakheri et al. 2010).

Reduction in relative water content under stress was evident through the experiments. In accordance to our results, drought induced subsidence of relative water content was reported (Ebrahimiyan et al. 2013). Higher relative water content in KCa-4884 in comparison to G-4 acknowledged to the ability of the genotype to retain water allowing plants to maintain hydrated leaves. Subsequently,

Fig. 1 Drought induced changes in net photosynthetic rate (a), stomatal conductance (b), sub-stomatal CO_2 concentration (c) and relative water content (d) among two cultivars. Values represent

mean $\pm$ SD. * denotes significant difference between tolerant and susceptible genotypes at 5% probability under drought stress conditions

hydrated leaves may be associated with better photosynthetic performance under dry conditions. Owing to the above assumption, KCa-4884 which conserved better leaf water status was revealed to retain relatively higher net assimilation under water deficit conditions.

Flow of water across the cell membrane is regulated by the activity and abundance of AQPs (Santos and Mazzafera 2013). Aquaporins not only provide low resistance pathway, they also control water movement across membranes as aquaporins are gated proteins (Bárzana et al. 2014a, b). Since regulation of AQP activity helps in maintaining water balance in plant and reverse transcription quantitative real time polymerase chain reaction (RT-qPCR) is widely used technology for gene expression studies, the

current work studied drought induced expressions of PIPs, NIPs, TIPs and XIPs. To accurately analyse RT-qPCR data, normalisation with a reference gene is prerequisite. In the present study, drought induced AQP gene expression was normalised with UBI-3 gene expression. According to Wan et al. (2011) reference gene Ubiquitin conjugating protein (UBI-3) showed high stability in pepper under abiotic stresses.

Owing to their location in plasma membrane, PIPs are considered as important AQPs functionally. When plants were subjected to drought stress, up-regulation of both PIP1-1 and PIP1-2 was observed in KCa-4884. Prolonged and increased drought stress resulted in up-regulation of the PIP genes investigated (Galmés et al. 2007). In drought

Fig. 2 Heatmap of drought induced relative gene expression of AQP genes in KCa-4884 and G-4

susceptible genotype G-4, both PIP1-1 and PIP1-2 were down-regulated. Down-regulation of PIPs would have been one of the reasons rendering susceptibility to G-4. Reduction of PIP expression reported to hasten wilting under water stress (Siefritz et al. 2002). There exists, considerable variation in drought induced PIP aquaporin expression depending on several factors such as tissue, species, variety, presence or absence of symbionts and severity of stress. Comprehensive studies in *Arabidopsis thaliana*, revealed transcriptional down-regulation of some PIPs under drought while other PIPs were found to be up-regulated (Jang et al. 2004; Alexandersson et al. 2005, 2008). In *Xenopus* oocytes, co-expression of PIP1 and PIP2 increased water permeability to several folds (Fetter et al.

2004). Augmentation of both PIP1-1 and PIP1-2 transcript abundance would prove to be beneficial in drought tolerant chilli genotype as they may be associated with improved hydraulic conductivity and photosynthetic pursuance.

Drought induced NIP expression was enhanced in KCa-4884 while it was abridged in G-4. Promoter regions of NIPs are enriched with drought responsive element MBS. This may induce differential expression of NIPs under drought. NIP4-1 is found to be highly regulated by drought (Liu et al. 2009). Furthermore, according to Ma and Yamaji (2008), NIPs (OsNIP2;1 and OsNIP2;2) in rice have ability to transport larger solute silicic acid that reinforce resistance of plants to abiotic stresses. Thus up-regulation of NIPs studied may have rendered drought tolerance in KCa-4884.

TIP subfamily members are known to assist water transport across vacuoles. Apart to their role as water channels, isoforms of TIPs are also capable of transporting glycerol, hydrogen peroxide (Maeshima 2001), ammonia (Kirscht et al. 2016) and urea (Liu et al. 2003). Differential regulation of TIP gene expressions affirmed their response to drought, abscisic acid (ABA) and salinity (Boursiac et al. 2005; Rodrigues et al. 2013; Regon et al. 2014). In the present study, down-regulation of TIPs was observed in G-4. OsNIPs expression is down-regulated under a variety of stresses, such as drought, salt, and cold (Liu et al. 2009). Whereas KCa-4884 revealed up-regulation of all TIPs. Increased expression levels of TIPs contributed tolerance to different abiotic stress factors (Wang et al. 2013). Also, in *Arabidopsis*, over expression of TIP1 conferred tolerance by impeding water loss when compared with wild under drought (Martinez-Ballesta and Carvajal 2014). In addition, drought tolerance has been conferred by over-

Fig. 3 Expression levels of 12 aquaporin genes in two pepper genotypes subjected to drought stress. *indicates significant different between two genotypes at 5% probability

expression of TIPs in transgenic plants (Peng et al. 2007; Khan et al. 2015; Li and Cai 2015). Persistent to the above statements, up-regulation of all TIPs would be reasons for drought tolerance in KCa-4884.

Uncharacterised X intrinsic proteins (XIPs) have been recognised in higher plants such as *Glycine max*, *Populus trichocarpa* and *Solanum lycopersicum* (Reuscher et al. 2013; Zhang et al. 2013). Solanaceae XIPs are found to be plasma membrane aquaporins involved in translocation of many uncharged substances such as urea, Hydrogen peroxide and Boric acid (Bienert et al. 2011). A significant drought induced up-regulation of XIPs was found in tolerant KCa-4884. According to Martins et al. (2015), XIPs were up-regulated in leaves of sweet orange when plants were rendered to drought treatment.

In the present study, drought tolerant KCa-4884 exhibited up-regulation of all studied AQP genes. Our results are in accordance with Reddy et al. (2017) who reported higher transcript abundance of AQPs in drought tolerant mulberry genotypes. All studied AQPs were down-regulated in G-4. Though general down-regulation of MIPs is way for plant to reduce water loss, up-regulation of individual MIPs is prerequisite for plant to channelize water flow to cells that are crucial for plant survival during stress (Alexandersson et al. 2005). Attributable to the above supposition, although expression of AQPs was reduced which would minimise water loss, due to less expression of individual AQPs, G-4 inclined towards drought susceptibility.

In conclusion, our study makes a significant contribution to the study of AQP genes during the response to drought by simultaneously measuring AQP gene expression in leaves and physiological parameters associated with water use efficiency of two chilli genotypes differing in tolerance to drought. This data shows that modulations of AQPs expression under drought stress conditions could be correlated to better photosynthetic performance. These results will allow us to understand physiological and molecular responses of chilli plants exposed to severe drought stress which could be used to improve drought tolerance in chilli.

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