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Received 4 April 2014; revised accepted 18 June 2014

Expression analysis of drought-induced genes in wild tomato line (*Solanum habrochaites*)

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Many plant genes are regulated in response to abiotic stresses such as drought, high salinity, heat and cold, and their gene products function in stress response and tolerance. The whole process of plant adaptation to these environmental stresses is controlled by orchestration of complex molecular networks. In the present study, eight genes showing significant difference of expression on exposure to artificial drought stress in tomato, were selected from the previously performed microarray experiment. Expression analysis of the genes was done semi-quantitatively as well as quantitatively under artificially imposed drought stress and the results were almost similar to those of microarray experiment. Tissue-specific analysis of the genes, performed on tolerant line, revealed fairly a similar pattern of expression in root, stem and leaf with notable differences in flower, which experienced the least influence of drought. The results confirmed that *SlPRP16*, *SlCYP51-17*, *SlMCPI19* and *SlGDSL20* were downregulated in both the lines with stronger downregulation in sensitive line. *SlWRKY4* was downregulated in both the lines with more folds of downregulation in tolerant line. *SlEFH12* and *SlSNF4-15* were upregulated in tolerant line. *SlUSPA9* was upregulated in both the lines with relatively more folds of upregulation in sensitive line.

Keywords: Abiotic stress, drought, gene expression, tomato, transcription factors.

TOMATO (*Solanum lycopersicum*), a major horticultural crop consumed all over the world, suffers heavy losses due to drought. Water deficit causes various physiological and biochemical effects on plant populations. In response, plants utilize a number of protective mechanisms to maintain normal cellular metabolism and prevent damage to cellular components. Tolerance to water stress in plants is generally associated with maintenance of plant water status. This is achieved through closing of stomata to reduce transpiration, enhancing the capacity of roots to extract more water from soil and osmotic adjustment by accumulating low molecular weight molecules. Drought response, being a complex signalling network, leaves a number of genes with upregulated expression and an equal number of genes with downregulated expression. Most of these upregulated and downregulated genes are directly or indirectly linked to each other.

WRKY transcription factors, earlier identified as key regulators of biotic stress, have been reported to impart abiotic stress tolerance in plants[1,2]. The role of WRKY transcription factors as negative regulators of abiotic stresses was revealed by constitutive expression of *BcWRKY46* gene in transgenic tobacco, under the control of the *CaMV35S* promoter, which conferred susceptibility of transgenic tobacco to freezing, ABA (abscisic acid), salt and dehydration stresses[3]. EF-hand proteins, with a helix–loop–helix Ca^{2+} binding motif, are one of the largest protein families involved in modulation of intracellular Ca^{2+} levels in response to various signals, including hormones, light, mechanical disturbances, abiotic stress and pathogen elicitors[4–7]. USP (universal stress protein) family proteins, first identified in prokaryotes, appear to play an active role in abiotic stress response, but their function remains largely unknown in plants. A USP gene (*SpUSP*), cloned from wild tomato (*Solanum pennellii*) and functionally characterized in cultivated tomato, exhibited increased expression under dehydration stress, salt stress, oxidative stress and phyto-hormone ABA treatment[8]. SNF1 (sucrose non-fermenting 1)/SNF1-related kinases/AMPKs (adenosine monophosphate-activated protein kinases) are evolutionary conserved sensors found in all eukaryotic organisms from simple unicellular fungi

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(yeast SNF1) to roundworms (AMPK), insects (AMPK), plants (SnRK1) and animals (AMPK). These protein kinases are important regulators of gene expression in response to energy or nutrient depletion stress conditions and, in some instances, regulate the activity of key metabolic enzymes[9].

PRPs (proline rich-proteins) contribute to cell wall structure of specific cell types and are involved in plant growth and development[10]. PRPs have been reported to accumulate in the cell wall in response to physical damage or other biotic and abiotic stress conditions[11–14]. Obtusifoliol 14α-demethylase, classified as CYP51 (cytochrome P_{450}), a member of the cytochrome P450 monooxygenases superfamily, is involved in post-squalene biosynthesis of sterols that serve as precursors for bioactive molecules such as mammalian steroid hormones, plant BR (brassinosteroid) hormones and insect ecdysteroids[15]. Brassinosteroids are the group of plant growth regulators known to affect a wide variety of physiological processes, including cell elongation, division, vascular differentiation, senescence and stress responses[16,17]. Metallocarboxypeptidases are an important class of enzymes that catalyse the hydrolysis of peptide bonds at the C-terminus of peptides and proteins, and play a key role in certain proteolysis-regulated physiological processes. MCPI (metallocarboxypeptidase inhibitor) inhibits the activity of metallocarboxypeptidases belonging to MEROPS peptidase family[18], but its role in abiotic stress response is still undiscovered. GDSL lipases/esterases play an important multifunctional role in plant growth, development, morphogenesis and have been found in various plant species, including *Arabidopsis*, rice and maize[19]. Enhanced expression of lipase and lipase-like genes was reported to be triggered by biotic and/or abiotic stresses such as pathogen infection, ethylene and salicylic acid treatment and UV-irradiation[20–22].

In the present study, we selected eight putative drought-responsive gene sequences from the earlier results of microarray experiment in our laboratory. Bioinformatics analysis of these sequences revealed that they encode for WRKY transcription factor, EF-hand containing protein, USP-A, SNF4 protein kinase, PRP, obtusifoliol 14α-demethylase, MCPI and GDSL esterase. Expression analysis by RT–PCR (reverse transcription PCR) and real-time PCR in the leaf tissues of tomato plant indicated that *SlWRKY4*, *SlPRP16*, *SlCYP51-17*, *SlMCPI19* and *SlGDSL20* genes are downregulated, while *SlEFH12*, *SlSNF4-15* and *SlUSPA9* genes are upregulated under artificially imposed drought stress. Tissue-specific expression study of the above-mentioned gene sequences was done with root, stem, leaf and flower.

Eight putative drought-responsive PROB SET IDs/gene sequences, which were either highly upregulated or downregulated under drought stress, were selected from the microarray experiment performed previously in our laboratory (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE22304) on drought-tolerant line (EC520061) of *Solanum habrochaites* and drought-sensitive line (CO3) of *Solanum lycopersicum* (Table 1). Probable ORF sequences for the selected sequences were deduced from FGENESH tool: HMM-based gene structure prediction (http://linux1.softberry.com/). Full-length gene primers and internal primers were designed for RT-PCR and real-time PCR analysis of the genes respectively (Table 2).

Seeds of the drought-tolerant and drought-sensitive lines were collected from the germplasm section of the Indian Institute of Vegetable Research, Varanasi. These seeds were sown in pots (30.0 cm diameter and 30.0 cm height) filled with a mixture of soil and compost. Germinated seedlings were maintained at 25°C under optimal conditions in a glass house with regular watering. To induce expression of the target genes, drought stress treatments were given to 3-month-old plants by withholding water for 14 days (Figure 1). After treatment, leaves were taken in three biological replications from drought-treated and control plants, frozen in liquid nitrogen, and stored at –80°C for further analysis. For tissue-specific expression analysis, samples were taken in three biological replications from root, stem, leaf and flower of tolerant line.

Total RNA was extracted from the leaves using TRI Reagent (Ambion) in combination with RNAase-free DNAase treatment (Qiagen) to remove contaminated DNA. The first-strand cDNA was synthesized by 1.0 µg of total RNA in 20 µl reaction volume, using first strand cDNA synthesis kit, according to the manufacturer's instructions (Bio-Rad).

RT-PCR was performed using 2 µl of the first-strand cDNA as template in 50 µl volume containing 36 µl H_2O, 5 µl 10× PCR buffer, 3 µl 25 mM $MgCl_2$, 1 µl 10 mM dNTP mix, 1 µl of each 10 mM sense and anti-sense primers and 1 µl *Taq* DNA polymerase (Fermentas Life Sciences). The PCR temperature programme was set as 1 cycle of 5 min at 94°C followed by 35 cycles of 30 sec at 94°C, 30 sec at 50–55°C (depending upon melting temperature (T_m) of primers) and 40 sec at 72°C, and finally 1 cycle of 10 min at 72°C. The resulting PCR fragments were electrophoretically separated on 1.2% agarose gels.

Real-time PCR was done using iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. Primers for all the target genes and α-tubulin gene were used (primers for α-tubulin – forward: CACTAGTGTCGCTGAGGTTTTCT and reverse: TGACCCGTCAAACTCTTACTCAT; product size = 240). The reverse transcription efficiency of target genes and α-tubulin gene was almost equal as analysed by comparing the cycle threshold (C_T) values at different dilutions of cDNA. All samples were amplified in triplicate and the mean value was considered. The C_T value is the number of cycles required to accumulate enough SYBR green fluorescent signal to exceed the threshold (background) level. The C_T value is proportional to the amount of real-time PCR product and was used for quantification. The

Table 1. Expression of selected genes from microarray experiment. The probe set IDs (assigned gene IDs) for each gene are indicated

Prob set IDs (assigned gene IDs)	Tolerant cultivar		Sensitive cultivar	
LesAffx.837.1.S1_at (*SlWRKY4*)	14.69221	down	4.195137	up
Les.4149.3.S1_at (*SlEFH12*)	13.51061	up	1.482424	down
Les.4233.1.S1_at (*SlUSPA9*)	24.34537	up	150.8232	up
Les.12.1.S1_at (*SlSNF4-15*)	13.64423	up	1.043047	up
Les.228.1.S1_a_at (*SlPRP16*)	17.67712	down	76.51508	down
Les.3094.3.S1_at (*SlCYP51-17*)	17.82661	down	154.9021	down
Les.506.1.A1_at (*SlMCPI19*)	21.30087	down	126.9683	down
Les.1079.2.S1_at (*SlGDSL20*)	23.06282	down	188.4652	down

The fold change (treated versus control) along with upregulation (up)/downregulation (down) in drought-tolerant and drought-sensitive lines of tomato is also represented.

Table 2. Primers for RT-PCR (full length gene primers) and real-time PCR (internal short primers)

Gene	Type	Full length gene primer sequence (5′–3′)	Product size (bp)	Internal primer sequence (5′–3′)	Product size (bp)
SlWRKY4	Forward	GCCATCACGGAATTCTCAG	999	CTCCCTCTGCTCATGATTCC	116
	Reverse	CCAAGAACATAGCCGAAGGT		AATGGCCTCAATTTCACCAA	
SlEFH12	Forward	TGAGTTGTGTTGACAAGGAATTAAA	1190	GGAGCAACTAACGAGCAACC	115
	Reverse	TCAGTTACATCTCAGAAATAGATGGAG		CGCTGTGAGCAACAATGACT	
SlUSPA9	Forward	AGAATACATGGAGGCGGAGA	520	GGACCTGCTGTGAGAAAAGG	102
	Reverse	TTGTTCATAGACTGGCACATGA		AATCAGAGTCTCCGCCTTCA	
SlSNF4-15	Forward	CAAACAAAATGCAGGCAACA	1167	ATTGCTGGAAACGGTTATCG	115
	Reverse	AAAACGGGTGCAAAAGAGTG		GGCTGAGGGTCCACAAACTA	
SlPRP16	Forward	TTCTTTTCATTTCTATGCTTTTCC	815	CAACAACAAAGGCAACATGC	94
	Reverse	ACATCCTTTCATGGCAATCC		GGATCACCAAGGCCAATATG	
SlCYP51-17	Forward	CAGGGATATTTTTCAGCTATGGA	1507	CTTTTGGTGGAGGCAGACAT	121
	Reverse	CCACATTCATGCAGATGTTATC		ATTTCAGGGAAAGGCGAGAT	
SlMCPI19	Forward	TTTTCACCATTTTCCTTGTGG	262	GCCCAAGATGCTGTTCTACC	108
	Reverse	TGAGCTTTCAAATCAATGCAA		CCGACAGGCTTCACAGTACA	
SlGDSL20	Forward	CATGATGAATTGCTCATTGTCTT	1140	GGATCCATTCCATCCATCTG	105
	Reverse	AAATGTTGAAACGTTGAGGAGAA		GCCATAATGGTGCTGAGGTT	

relative value obtained for quantitation was expressed as $2^{-\Delta\Delta C_T}$ where ΔC_T represents the difference between the C_T value of the sample and that of α-tubulin (endogenous control) and $\Delta\Delta C_T$ is difference between the ΔC_T value of a sample and that of its respective control[23].

The selected gene sequences were searched for the corresponding ORF sequences using FGENESH tool: HMM-based gene structure prediction (http://linux1.softberry.com/). Conserved domain search was performed with the already deduced ORF sequence of each gene in 'Conserved Domain Database' (CDD) of NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) for determining the function of selected genes. BLASTn search was performed with NCBI BLASTn tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to find the functional similarity of selected genes with the those of other plant species. Subcellular localization for the predicted plant protein was confirmed by 'ProtComp 9.0' tool (http://www.softberry.com/berry.phtml?topic=protcomppl&group=programs&subgroup=proloc). Chromosome location of genes was determined using Sol Genomics Network BLAST tool (http://solgenomics.net/tools/blast/index.pl) (Table 3).

Expression analysis of all the genes was done semi-quantitatively using RT-PCR (Figure 2) as well as quantitatively using real-time PCR (Figure 3) in both the lines (tolerant and sensitive) under artificially imposed drought stress. The real-time PCR expression pattern matched the expression pattern of the microarray experiment with slight differences in a few cases. Real-time PCR analysis showed that *SlWRKY4* gene was downregulated in both the lines as against the microarray expression data, where it was slightly upregulated in sensitive line. Semi-quantitative expression analysis by RT-PCR showed faint bands of *SlMCPI19* gene under drought stress in both the lines, while its expression was substantial under control condition. RT-PCR analysis of *SlUSPA9* gene indicated its negligible expression under control conditions in both the lines, but the gene was significantly expressed in drought-treated samples.

Tissue-specific (root, stem, leaf and flower) expression analysis was performed using real-time PCR in a tolerant line under artificially imposed drought stress (Figure 4). Majority of genes revealed similar pattern of expression in root, stem and leaf, except *SlPRP16* gene which

Figure 1. Drought-tolerant and drought-sensitive lines of tomato maintained at 25°C under optimal conditions in a glass house. ***a***, Tolerant line under normal water condition; ***b***, Tolerant line under artificial drought stress; ***c***, Sensitive line under normal water condition; ***d***, Sensitive line under artificial drought stress.

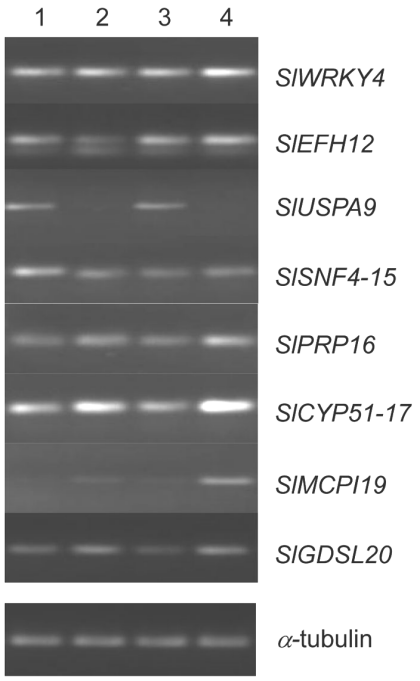

Figure 2. RT-PCR expression bands of target genes along with endogenous control (α-tubulin). Lane 1, Treated (tolerant line); lane 2, Control (tolerant line); lane 3, Treated (sensitive line); lane 4, Control (sensitive line).

documented 3420 fold downregulation in the root, indicating the partial switch-off of PRP in root tissues under drought. There was no major effect of drought stress on the expression level of all the genes in the flower except *SNF4-Sl-15* which exhibited about 13 fold downregulation.

SlPRP16, *SlCYP51-17*, *SlMCPI19* and *SlGDSL20* genes encode for metabolically important proteins like proline-rich cell wall protein, obtusifoliol 14α-demethylase, metallo carboxypeptidase inhibitor and GDSL esterase. They were downregulated under drought stress in both the lines with considerably more downregulation in the sensitive line. This indicates that during drought, the plant tends to suppress the expression of these genes directly or indirectly. WRKY transcription factors are commonly reported to play a positive role in biotic as well as abiotic stresses in various plant species[1,2]. However, recently, it was observed that constitutive expression of *BcWRKY46* gene in tobacco induced susceptibility to freezing, ABA, salt and dehydration stresses[3]. In the present study, *SlWRKY4* gene was downregulated by drought in both the lines with relatively more downregulation in the tolerant line.

USPs appear to play an active role in abiotic stress responses, but their function is still ambiguous in plants. Two *Arabidopsis* USP genes, *At3g62550* and *At3g53990*, that encode an ATP-binding motif, were upregulated in a drought microarray dataset[24]. Tomato plants overexpressing *SpUSP* gene, cloned from wild tomato (*S. pennellii*), accumulated high concentration of ABA and exhibited increased drought tolerance in seedling and adult stages, possibly because high ABA concentration induced stomatal closure and thereby reduced water loss[8]. In another experiment, microarray data revealed that a large number of chlorophyll *a*/*b* binding proteins were also upregulated in *SpUSP* overexpressing tomato plants[8]. Thus, it was concluded that USP guided the stomatal closure through ABA and maintained the photosynthetic functions. In the present study, negligible expression of *SlUSPA9* gene under control conditions, as revealed by RT-PCR analysis, indicates that the gene is strictly regulated by drought stress.

EF-hand proteins typically contain a Ca^{2+} binding domain and their expression is induced by drought, ABA and high salinity[4–7]. In the present study, expression of

Table 3. Chromosome localization of gene, predicted subcellular localization of protein and predicted function of selected genes

Gene	Location on chromosome	Subcellular location of protein	Predicted function
SlWRKY4	03	Nuclear	WRKY family transcription factor/DNA-binding protein (WRKY4) of *Nicotiana tabacum*
SlEFH12	01	Cytoplasmic, chloroplast	EF-hand containing protein of *Solanum tuberosum*/calcium-binding EF hand family protein
SlUSPA9	01	Cytoplasmic, peroxisome, extracellular	Universal stress protein A of *Arabidopsis thaliana*/universal stress protein (USP) family
SlSNF4-15	06	Cytoplasmic, chloroplast	SNF4 protein kinase of *Solanum lycopersicum*/CBS domain-containing protein/AMP-activated protein kinase
SlPRP16	12	Extracellular, plasma membrane	Proline-rich cell-wall protein of *N. tabacum*/protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
SlCYP51-17	01	Plasma membrane, mitochondrial	Obtusifoliol 14α-demethylase of *S. lycopersicum*/cytochrome P450 mono-oxygenase/abscisic acid 8′-hydroxylase
SlMCPI19	07	Extracellular	Metallocarboxypeptidase inhibitor IIa of *S. lycopersicum*
SlGDSL20	04	Extracellular, vacuolar, chloroplast	GDSL esterase/lipase/acyl hydrolase/fatty acyl transferase/Zn finger protein of castor

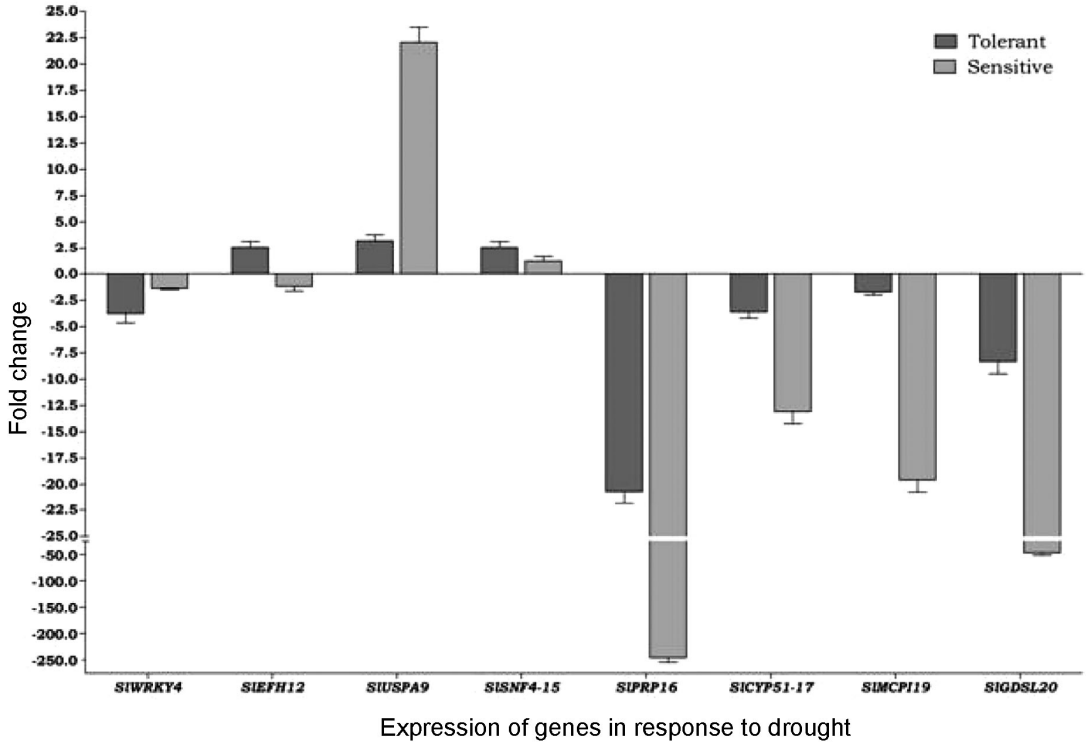

Figure 3. Quantitative expression of genes in leaves of tolerant and sensitive lines of tomato under artificially induced drought stress. On the *y*-axis, the negative values represent downregulation and positive values represent upregulation.

SlEFH12 was induced by drought in the tolerant line, signifying the gene as an important target for developing drought-tolerant transgenic plants. SNF4 is the regulatory gamma (γ) subunit of heterotrimeric complex that makes functional SNF1 protein kinase, the master regulator of the energetic and metabolic state of the cell[9]. SNF4 protein kinase of tomato (*LeSNF4*) was earlier reported to be induced in response to ABA and dehydration[25]. In the present study, expression of *SlSNF4-15* was induced by drought stress in both the lines, assuring its positive role in drought tolerance.

The influence of drought stress, as elucidated by tissue-specific expression analysis, was lowest for the flower. Exceptionally, *SlSNF4-15* gene, encoding for SNF4 protein kinase revealed maximum change of expression in flower tissues. It exhibited around 12-fold downregulation in flower tissues, compared to its upregulation in the rest of the tissues. Expression of *SlMCPI19* was not

Table 4. Real-time PCR data of *SlMCPI19* and *SlUSPA9* genes in root, stem, leaf and flower. Data indicate that accumulation of *SlMCPI19* and *SlUSPA9* RNA is more in flower tissues in both treated and control samples

Gene		Treated	Tubulin	ΔCT	Control	Tubulin	ΔCT	ΔΔCT	Fold changes
SlMCPI19	Root	33.84	17.37	16.47	31.67	16.95	14.72	1.75	0.297302
	Stem	34.07	16.68	17.39	31.01	17.84	13.17	0.22	0.858565
	Leaf	33.04	16.73	16.31	30.43	17.88	12.55	3.76	0.073812
	Flower	*23.48*	16.62	6.86	*21.74*	14.81	6.93	–0.07	1.049717
SlUSPA9	Root	22.67	17.32	5.35	24.52	17.64	6.88	–1.53	2.887858
	Stem	24.12	16.48	7.64	25.25	16.76	8.49	–0.85	1.802501
	Leaf	21.18	16.54	4.64	23.39	18.17	5.22	–0.58	1.494849
	Flower	*16.11*	15.84	0.27	*16.6*	15.98	0.62	–0.35	1.274561

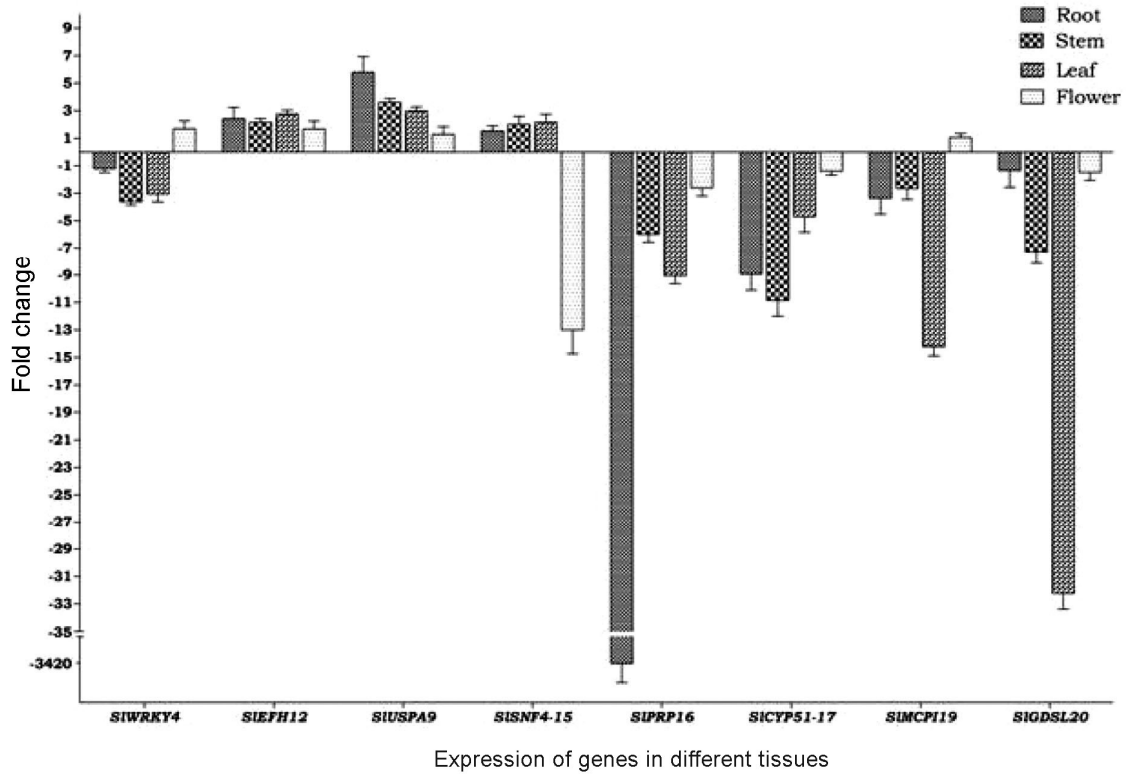

Figure 4. Quantitative expression of genes in root, stem, leaf and flower of drought-tolerant tomato line. On the *y*-axis, the negative values represent downregulation and positive values represent upregulation.

significantly altered by stress treatment in flower tissues, indicating the constitutive expression of *MCPI* gene in reproductive parts of tomato plant. Moreover, real-time PCR data revealed significantly high accumulation of MCPI RNA in flower tissues compared to root, stem and leaf (Table 4). Similar findings, i.e. high levels of MCPI RNA at anthesis stage ovaries, were earlier reported in tomato[26]. Like *SlMCPI19*, *SlUSPA9* gene also exhibited highest accumulation of RNA in flower tissues compared to root, stem and leaf (Table 4), but its expression in flower was least altered by stress treatment. The expression analysis of *SbPRP* gene demonstrated its accumulation in leaves and epicotyls of soybean seedlings, but not in cotyledons, hypocotyls and roots[11]. In the present study, drought-induced downregulation of *SlPRP16* gene in root tissues, confirms the negligible occurrence of PRP in root cell under drought.

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ACKNOWLEDGEMENTS. We thank Dr P. S. Naik, Dr Shailesh Tiwari, Dr H. C. Prasanna and Dr Suresh Reddy (Indian Institute of Vegetable Research (IIVR), Varanasi) and Dr Sanjeev Kumar (Indian Institute of Sugarcane Research, Lucknow) for useful comments and suggestions. Financial assistance for this work was provided by IIVR, Varanasi.

Received 21 December 2013; revised accepted 6 June 2014

Increase in agricultural patch contiguity over the past three decades in Ganga River Basin, India

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Ganga River Basin (GRB) is the second most populous river basin in the world, which has been undergoing rapid land-use change during the last few decades. Here, we analyse the landscape dynamics in Indian GRB (IGRB) using three indices, i.e. class area, mean patch size and number of patches for 14 land-use and land-cover (LULC) classes using multi-temporal Landsat satellite datasets of 1975 and 2010. Major change was observed with the expansion of agricultural lands and human settlements and depletion of

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