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A Polyketide cyclase/dehydrase and lipid transport superfamily gene of Arabidopsis and its orthologue of chickpea exhibit rapid response to wounding

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Abstract

In order to understand the mechanism of stress tolerance it is imperative that we identify factors that govern upstream reactions of stress signalling pathways for multiple stress tolerance. In this study, we have identified a gene, *AT5G54170*, from Arabidopsis and its orthologue in chickpea, which show rapid response to mechanical wounding. *AT5G54170* and its orthologue in chickpea (LOC 101512743) most likely plays a role in the jasmonic acid signalling pathway and the gene is also upregulated in several other stresses making it an ideal candidate for a primary stress-responsive transcript that is involved in the core stress response. Further characterisation of this gene and its promoter, along with its chickpea homologue, could provide valuable information on jasmonic acid signalling pathway and also explore their utility towards engineering stress tolerance in plants.

Key words: Wound response, signalling, START domain, stress

Introduction

Plants, being sessile, have evolved mechanisms to perceive signals to allow optimal response [1] to a variety of biotic and abiotic stresses. The responses to stress in plants are regulated by the action of phytohormones such as jasmonic acid, salicylic acid, ethylene, and abscisic acid [1]. These responses are largely constituted by the dramatic changes in the transcriptional machinery driven by the external stimuli. Large-scale transcript profiling studies have been successfully implemented to studying both abiotic and biotic stresses [2-4] and also combination of stresses in *Arabidopsis thaliana* [5]. These studies have shown that biotic and abiotic stresses trigger the expression of different but overlapping suites of genes [1, 6]. Initially, a core set of multi-stress-responsive genes are expressed, which progressively becomes stress-specific with the passage of time [4, 7].

Walley *et al*. [4] through a study have shown that a novel *cis*-element was responsible in driving biotic and abiotic stress responses when plants were subjected to mechanical injury. This *cis*-regulatory element was termed the rapid stress response element (RSRE) since the genes were induced within 5 minutes of wounding and this response was called the rapid wound response (RWR). The promoters of genes that quickly respond to wounding could be used to study their usefulness in the production of synthetic wound-inducible promoters to confer stress-tolerance to crop plants through genetic engineering [8]. In our study, we selected five genes from *A. thaliana,* which responded to wounding within 5 min of administration of the stimulus and chose one gene that seemed to respond to several stresses. The homologue of this gene, At5G54170, in chickpea was also identified and found to respond to wounding.

Materials and methods

In silico analysis

The Arabidopsis genes considered for this study were retrieved from the TAIR 9 database. Genevestigator [9] (https://www.genevestigator.com/gv/index.jsp) and geneMANIA [10] (www.genemania.org) were used for expression and co-expression analyses, respectively.

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TBLASTN was used for the identification of chickpea homologues using Arabidopsis protein as query sequence. Augustus [11] (http://bioinf.uni-greifswald.de/augustus/) gene prediction software was used for gene prediction. MUSCLE [12] was used for multiple sequence alignment and MEGA (ver 6) [13] was used to reconstruct a maximum-likelihood phylogenetic tree with the following parameters: JTT model with 100 bootstrap values. The transmembrane domains were predicted with the Kyte Doolittle Hydropathy plot [14]. The promoter *cis*-elements were analysed using PlantCare [15] and the domains were predicted using the Pfam database [16].

Plant material and stress treatments

Fifteen-day-old seedlings of Arabidopsis ecotype (Col) were used for the wound stress treatment. Surface sterilized seeds were sown in MS media and kept in 3 days under 4°C. After 3 days, plates were transferred to culture room for germination. fifteen days after sowing (DAS), wound stress was applied to the seedlings through forceps, seedlings were collected after different time intervals, frozen in liquid nitrogen and stored at –80°C. Chickpea desi variety ICC4958 was used in this experiment. Samples for five different tissues were collected from the field. Line sowing was done in the field during third week of October, 2013. During the pod development stage, shoots, roots, flowers and pods were collected from the field and immediately frozen in liquid nitrogen and stored in –80^{o}C for RNA isolation. For wound stress, pot sown fifteen day-old chickpea seedlings were used. The pots were kept in glass house maintained at 22±2^{o}C with 14 hours light condition. At 15 DAS, the plants were subjected to wound stress with the help of forceps and seedlings at different time points *viz*., 0 min, 15 min, 1 hour, and 6 hour after wound stress were collected, rapidly frozen in liquid nitrogen, stored at –80^{o}C for RNA isolation.

RNA isolation, cDNA synthesis and Real time PCR

Total RNA was isolated by Trizol method. The integrity and quality of RNA was analyzed through agarose gel (1% w/v) and nano drop ND-1000 UV-Vis spectrophotometer respectively. 10 µg of RNA was used for the DNase I treatment and 1µg DNase treated RNA was used for the first strand cDNA synthesis using superscript III Reverse transcriptase (Invitrogen). Quantitative Real Time PCR was performed in step one plus instrument (ABI). Three biological replicates with three technical replicates were used for each tissue and wound stress samples. GAPDH and Act8 genes were used for normalization of cDNA. Each reaction contained 5 µL 2xSYBR Master mix reagent (Genetix), 1uL cDNA and 200 nM of gene specific primers in a final volume of 10 µL. Each pair of primers was designed using the Primer3plus program with an amplicon size ranging from 150-200bp (Table 1). The thermal cycle used is as follows: 95^{o}C for 10 min for activation, 40 cycles of 95^{0}C for 15s for denaturation and 60^{o}C for 1min for annealing and extension. The specificity of reaction was analyzed using melting curve analysis. The amplified products were also visualized by electrophoresis in a 1.5 % agarose gel to verify the expected fragment size. The relative transcript level of the gene for different samples was determined by ΔΔCT method in comparison with the shoot values. For wound stress, 0 min was considered as control and fold change was calculated by a similar method.

Table 1. Primer sequences used in the study

Gene	Forward/ reverse	Sequence
Chickpea primer sequences		
LOC101512743	FP	TCCACTCTCCCGTTCAG
	RP	CGCCCTGTGGTTTATCAG
GADPH	FP	ACCTACGACGAAATCAAGG CTGCT
	RP	ACAATGAGGTCAACGACAC GGGTA
Arabidopsis primer sequences		
ACT8	FP	ATGAAGATTAAGGTCGTGGCA
	RP	GACATCTCTCCAAACGCTGT
AT1G72920	FP	ATGTTTTAACTGTTGGTT GAAGG
	RP	TTTCTCAGTGAAATTCGGG TTATT
AT2G20340	FP	CTTCATGTCGCACACTGC TTTAT
	RP	GCTTCTTCCTGAATAATC TCCAC
AT2G34810	FP	ATGTCGATATCGGAATTAC TACGC
	RP	GGGCTTTAATCTTAACCAA TCTCA
AT5G47240	FP	CTACTTTTGATGGCAAACC TTCTT
	RP	TTTGGTTCTGGTTTACCCG TCTAT
AT5G54170	FP	AGAAACTGTTGATTATCGG TGGAG
	RP	CATTAACAAACCTTTTCCC AAAAC

Results and discussion

Expression analysis through semi-quantitative RT-PCR and quantitative real time - PCR

The TAIR Gene Search (TAIR9) page was accessed and in the column “Search by Name or Phenotype” a search was conducted with the term ‘wound’ contained in the description of the genes. This search resulted in 42 loci matches and all of these gene sequences were retrieved. From this list we removed all the genes that were characterised and shortlisted the genes that were either hypothetical or unknown proteins. A semi-quantitative RT-PCR was done for the genes chosen to study their expression levels in Arabidopsis five minutes after wounding. Walley *et al*. [4] studied the response of genes five minutes after wounding and opined that the rapid wound-responsive genes likely represented the initial components of the general stress response (GSR) or the cellular stress response that has been widely studied in animals and yeast and key molecular components of the GSR in organisms are evolutionarily conserved [17]. Several stresses trigger the GSR in a transient manner. The top five genes that showed maximum upregulation after wounding in the semi-quantitative RT-PCR study (Fig. 1) were validated for rapid wound response using quantitative Real Time–PCR (Fig. 2). The five genes were AT1G72920, AT2G20340, AT2G34810, AT5G47240, and AT5G54170. The maximum upregulation of 11-fold was observed in AT2G20340, while AT5G54170 was upregulated about 10-fold, AT1G72920 and AT2G34810 by 6-fold, and AT5G47240 by 4-fold. At the time of the experiment, AT2G20340 was predicted as tyrosine decarboxylase, which was later proved experimentally by Gutensohn *et al*. [18] as an aromatic aldehyde synthase. This enzyme catalyses the conversion of phenylalanine and 3,4-dihydroxy-L-phenylalanine to phenylacetaldehyde and dopaldehyde, respectively. An increased emission of phenylacetaldehyde due to feeding of foliage by *Pieris rapae* larvae was observed as a defensive mechanism against herbivores.

The second gene that showed 10-fold upregulation, AT5G54170, is a protein containing the lipid-binding domain-START (StAR-related lipid transfer), named after the 30 kDa steroidogenic acute regulatory (StAR) protein in mammals that help in the binding and transfer of cholesterol to the inner mitochondrial membrane [19]. AT1G72920 belongs to the TIR-NBS class of disease resistance proteins that have recently been implicated in plant basal defence

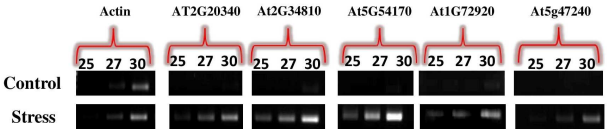

Fig. 1. Semi-quantitative RT-PCR of five putative wound-responsive genes from Arabidopsis at 25, 27, and 30 numbers of thermal cycles of untreated plants and plants harvested 5min after mechanical injury. (Top row-control; bottom row-stress)

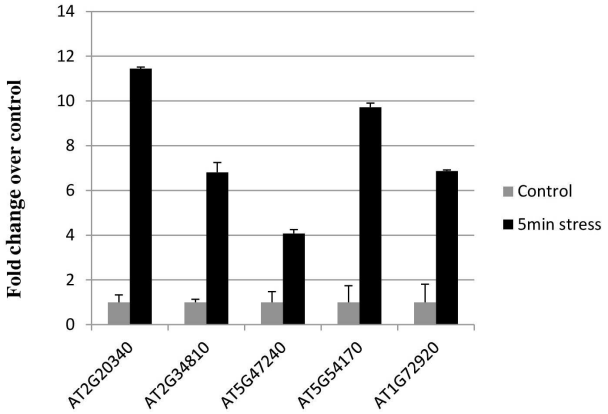

Fig. 2. Quantitative PCR analysis of candidate Arabidopsis genes in response to 5 min after wounding stress. Actin gene was used for normalization. The expression level of genes in control sample was used as calibrator to calculate fold change in expression

response [20]. AT2G34810 is a flavin adenine binding berberine family protein and AT5G47240 is annotated as NUDT8/Nudix hydrolase homolog 8. Therefore, of the five genes one has been characterised, while the other four have been annotated with putative functions.

Expression profiling using Genevestigator

The expression profiles of Arabidopsis genes under different perturbations, tissues, and developmental stages are available for public use on the Genevestigator database. In order to find the response of these selected genes to an array of stresses, Genevestigator was used. The filters used were high at a fold change of 2 and a p-value of <0.001 (Fig. 3). The resulting heatmap created using the “Perturbations” option in Genevestigator shows that the gene AT5G54170 is expressed at higher levels relative to the other genes. It shows upregulation in several stress conditions such as drought, salt, heat, and biotic

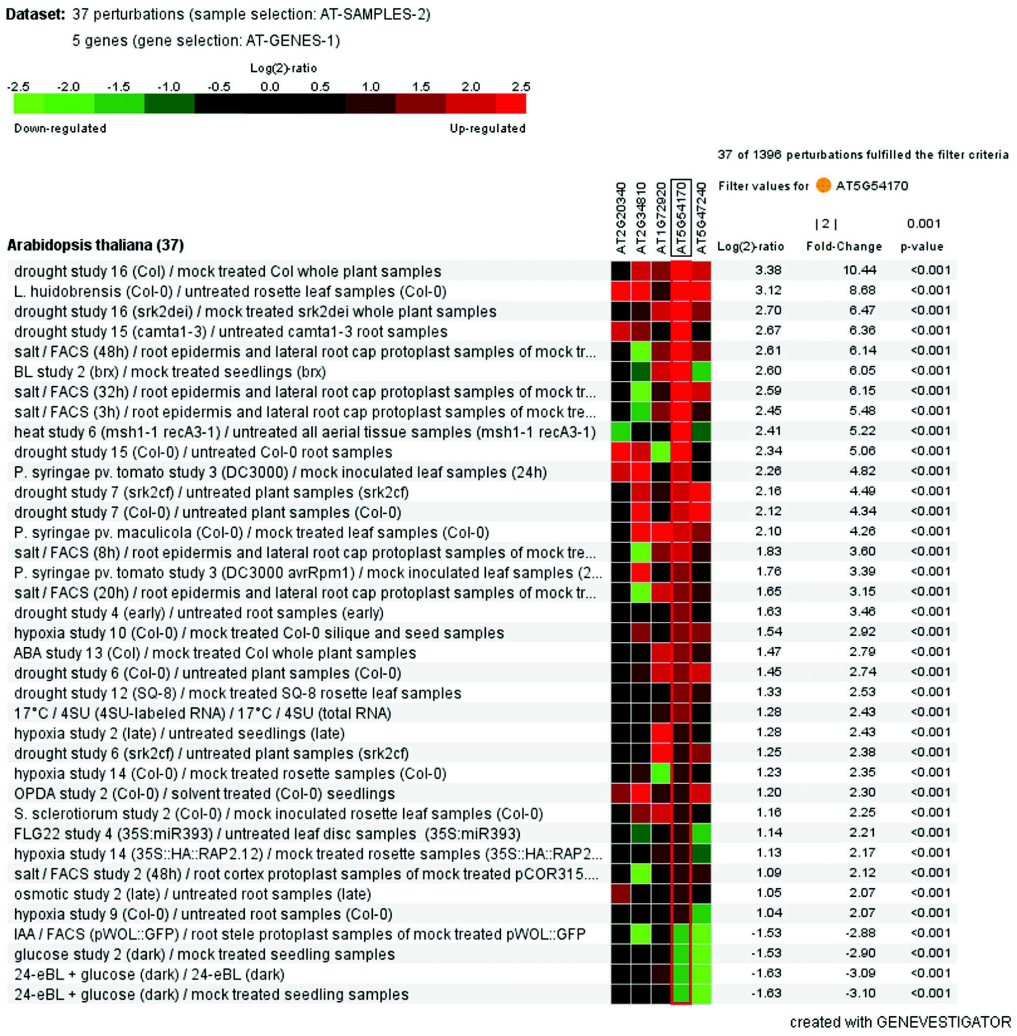

Fig. 3. Expression analysis of the Arabidopsis genes under different stress conditions using publicly available data on Genevestigator. Selection criteria used were: expression at 2-fold or more and p-value < 0.001

stresses among others. The expression of the five genes in the various tissues and developmental stages are shown in Fig. 4. Results from the expression analysis suggest that AT5G54170 is a likely player in the initial core response or the GSR. Therefore, we went ahead with further studies on this gene.

Involvement of AT5G54170 in the jasmonic acid signalling pathway

Genes showing similar patterns of expression may be involved in a similar response/pathway under stresses. Through co-expression analysis, the genes that are expressed along with the gene of interest with high correlation could provide us with information on the pathway the genes are involved in. A co-expression analysis run on GeneMANIA showed that the expression levels of the genes *AOS* (Allene oxide synthase) and *JAZ2* (Jasmonate-ZIM-Domain protein2) were strongly correlated with that of AT5G54170. *AOS* and *JAZ2* have been implicated in the jasmonic acid signalling pathway [21, 22]. Yan *et al*. [23] showed that both *AOS* and AT5G54170 were both upregulated by wounding. In an *aos*-mutant of Arabidopsis, where jasmonic acid accumulation is impaired in both resting and wounded leaves, AT5G54170 was downregulated. This clearly suggested that this gene may be involved in the jasmonic acid signalling pathway. Since the AOS protein is targetted to the chloroplast [24], we predicted the localisation of the AT5G54170 protein using the ChloroP server. The protein was indeed predicted to localise in the chloroplast. Since AT5G54170 is a START-domain (lipid binding) protein, we opined that the protein might be involved in lipid transport across the chloroplast membrane. To verify this, the hydrophobicity throughout the length of the protein was visualised with the help of a hydropathy plot (Fig. 5a). There were two distinct regions on the protein which displayed transmembrane domains, one at the N-terminal and the other at the C-terminal region.

Identification of the homologue of AT5G54170 in chickpea

Using the protein sequence of AT5G54170 as query, a tBLASTn search was carried out against the chickpea genome at NCBI. This identified two contigs,

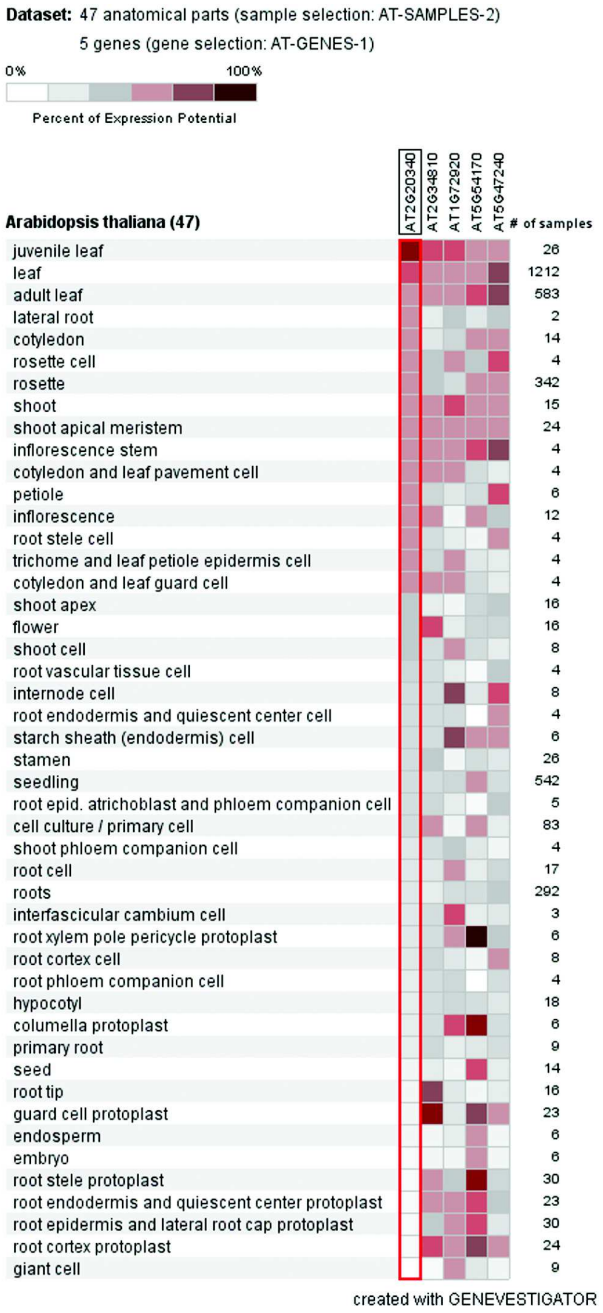

Fig. 4. Expression analysis of the genes in different tissues of Arabidopsis using publicly available data as viewed through Genevestigator

AHII0105716 and AHII01002750, with significant E-value. Using the gene prediction software AUGUSTUS, genes were predicted in the contigs. There were more than one gene predicted from both the contigs. LOC101512743 was one of the genes predicted from contig AHII0105716 and LOC101494889 was predicted from AHII01002750. LOC101512743 is an uncharacterized protein of 425 amino acids showing

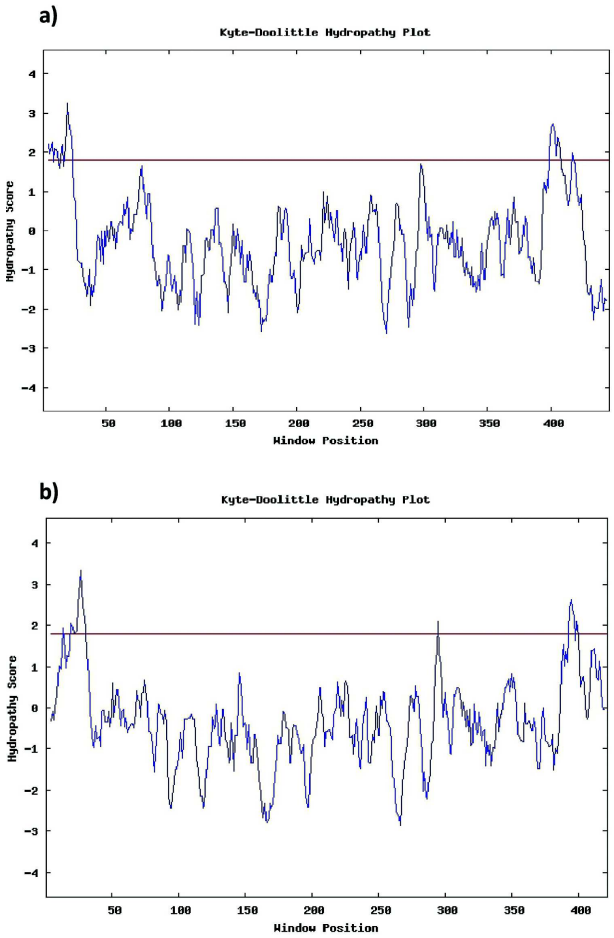

Fig. 5. Hydropathy plot of protein sequence a) AT5G54170 b) LOC10152743 showing a score of more than 2 indicating the presence of transmembrane domains in the amino- and carboxy terminal regions (Threshold value: 1.8 denoted by red line)

50.82% identity with AT5G54170, while LOC101494889, also an uncharacterized protein 399 amino acids long, was 56% identical to AT5G54170. Proteins predicted from these two genes were found to possess a START domain (Pfam) and two transmembrane domains as predicted with the hydropathy plot. The hydropathy plot for LOC101512743 is shown in Fig. 5b. The proteins were then used as queries for a reciprocal-blastp search on NCBI against the Arabidopsis refseq database. Both the proteins, LOC101512743 and LOC101494889, returned a 63% match with AT1g64720. AT1G64720 is a paralogue of AT5G54170 in the Arabidopsis genome, and is a membrane-associated protein that is localised to the vacuolar membrane [25]. Since a reciprocal-blast search was not useful in the

determination of homology of the proteins, we resorted to reconstructing a phylogenetic tree with all the Arabidopsis START domain containing proteins along with the two proteins that were predicted from the chickpea genome. The Maximum likelihood method of phylogenetic tree reconstruction under the Jones-Taylor-Thornton (JTT) model was adopted. It was clear from the phylogenetic reconstruction that the LOC101512743 was closer to AT5G54170 than the LOC101494889, which is the probable homologue of AT1G64720 as inferred from the tree.

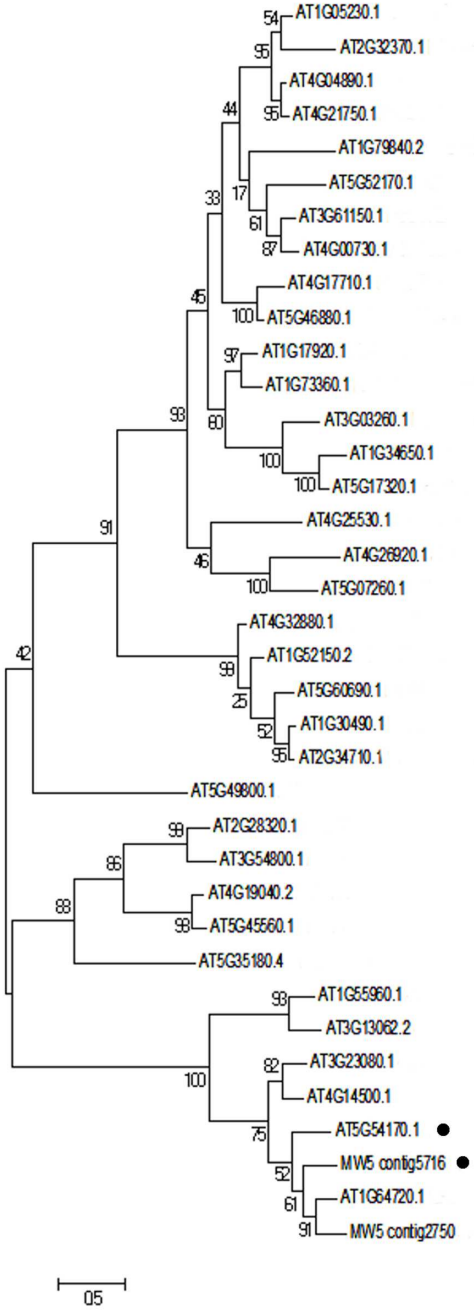

Fig. 6. Maximum-likelihood phylogenetic analysis of chickpea homologues of AT5G54170 with all the START domain containing proteins of Arabidopsis. Black dots indicate the Arabidopsis and chickpea homologues

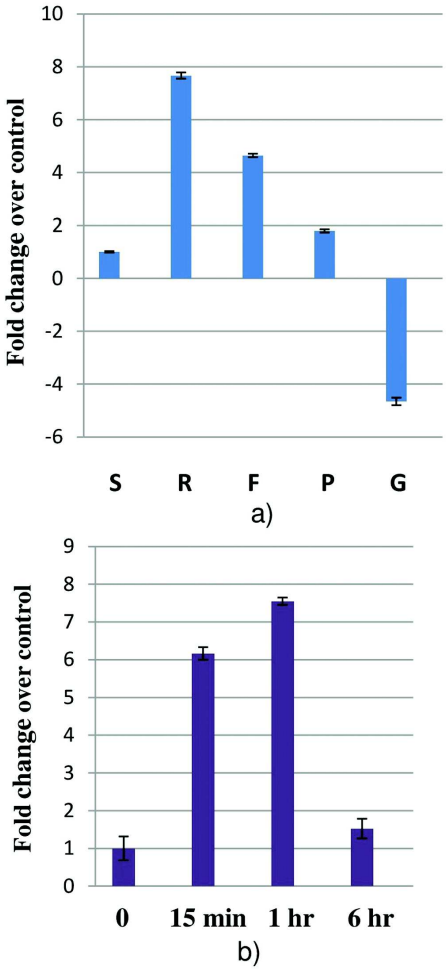

Fig. 7. Quantitative RT-PCR analysis of chickpea LOC101512743 in different tissues and wound stress. GADPH was used for normalization. The expression level of the gene in the shoot and 0 min of wound stress was taken as 1. S-Shoots; R-Roots; F-Flower; P-Pod wall; G-Grain. 0-Control, 15min, 1 hour and 6 hours are different time points of sample collection after stress treatment

Expression analysis of LOC101512743 in chickpea

The expression pattern of LOC101512743 was studied in five different tissues *viz.,* shoot, root, flower, pod wall, and grain, and under wounding stress at four time intervals, 15 min, 30 min, one hour, and six hour in seedlings. Compared to shoot and pod wall, the expression of LOC101512743 was higher in flower and

Table 2. *cis*-elements found in the promoters of LOC101512743 and AT5G54170

Cis-element	Sequence	LOC101512743	AT5G54170	Function of element
ACE	ACGTGGA	-	+	Light responsiveness
AE-box	AGAAACAA	-	+	Light responsiveness
ARE	TGGTTT	+	+	Anaerobic induction
Box 4	ATTAAT	+	-	Light responsiveness
Box-W1	TTGACC	+	+	Fungal elicitor responsive element
CGTCA-motif	CGTCA	+	+	MeJA responsiveness
G-Box	CACGTG/T	+	-	MeJA and light responsiveness
GARE-motif	AAACAGA	-	+	gibberellin-responsive element
HSE	AGAAAATTCG	+	+	Heat stress responsiveness
MNF1	GTGCCC(A/T)(A/T)	+	-	Light-responsive element
Skn-1_motif	GTCAT	+	+	required for endosperm expression
TC-rich repeats	ATTCTCTAAC	+	+	Stress- and defence - responsiveness
TCA-element	CAGAAAAGGA	-	+	Salicylic acid response
TGA-element	AACGAC	-	+	Auxin-response element
TGACG-motif	TGACG	+	+	MeJA responsive
W box	TTGACC	+	+	binding site for the WRKY family of transcription factors
box S	AGCCACC	-	+	Wounding and pathogen response

roots. The expression was the lowest in grains. Wounding stress upregulated the gene to more than 6-fold at 15 min after wounding and 7.5-fold at one hour after wounding. The *cis*-elements of the two genes, AT5G54170 and LOC101512743 , were also studied for the presence of stress-inducible elements (Table 2). Several motifs that play a role in hormone responsiveness including methyl jasmonate, and stress responsiveness are found to be present in the promoters of both the genes. Consistent with the stress responsive *cis*-element found in the promoter, abiotic stresses such as salinity and drought stress also appears to regulate the expression of LOC10152743 as their mRNA was detected in drought stressed SSH library (GenBank: HO064690) [26] and salt stressed root cDNA library (GenBank: GRK07965) [27]. This promoter can be further characterised for their wound- and abiotic stress-responsiveness and further utility in the development of synthetic promoters.

Our efforts have led to the identification of two wound inducible genes, homologues from Arabidopsis and chickpea, which show rapid wound response and may play a key role in the jasmonic acid signalling pathway. Further characterisation of these genes and their promoter sequences would provide us more insights into the pathways they are involved in. If these genes are found to be involved in upstream processes of stress pathways, they will make for good candidates for engineering multi-stress tolerance in plants.

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