Chapter 3

Study of touch induced TCH gene orthologues in *Cajanus cajan*

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3.1 Abstract:

Cytosolic calcium (Ca $^{2+}$) levels is altered upon mechanical perturbations. Ca^{2+} mediated intracellular communication is generally facilitated by calcium sensors; calmodulin (CaM) and calmodulin like-molecules (CML). Touch induced CaM and CML also as known as TCH genes. In our work we have attempted to identify *C. cajan* counterparts of three Arabidopsis TCH genes namely *AtTCH1* (calmodulin; CaM2), *AtTCH2* (CML24) and *AtTCH3* (CML12). Expression of the two *AtTCH* gene orthologue (*CcTCH1-1* and *CcTCH2-2*) were found to be upregulated at an early time point after touch treatment. This study indicates presence of conserved molecule for touch response in *C. cajan*.

3.2. Introduction:

Under normal conditions, the cellular calcium levels are maintained low by sequestering them in endoplasmic reticulum (ER). The Ca^{2+} ions are released into the cytosol and levels are increased in response to various biotic and abiotic stress conditions. Ca^{2+} mediated signalling is triggered by release of Ca^{2+} from either ER or extracellular spaces. Ca^{2+} reflux is also triggered by plant defense hormones like salicylic acid during pathogen infection (Blume et al 2000; Grant et al 2000) as well as upon mechanical stimulations like touch, herbivore attack and wounding in plant (Cosgrove and Hedrich 1991; Batiza et al 1996; Calaghan and White 1999). The change in Ca^{2+} is sensed by cytosolic calcium sensors like calmodulin (CaM) and calmodulin like-molecules (CML). Pathogen triggered variations of intracellular Ca^{2+} levels triggers activation of CaMs in *Nicotiana* (Heo et al 1999). Isoforms of CaMs that are activated during plant immune response are pathogen or elicitor specific. CaMs are also known to regulate plant immunity by regulating biosynthesis of plant defense hormone SA (Du L et al 2009). Hypersensitive reaction in response to pathogen in tobacco plants also involves calcium triggered apoptosis (Levine 1996; Grant et al 2000). Studies performed in Arabidopsis shows that many CaM and CML are expressed at early time point after touch stimulation (Braam and Davis 1990; Lee et al 2005). The major calmodulin protein is TCH1 (CaM2) and CML proteins like TCH2 and TCH3 respectively. In Arabidopsis there are seven CaM molecules and only TCH1 was found to be up-regulated upon touch stimuli (Lee et al 2005).

Increased levels of calcium sensors in both touch and stress response indicates that there is a common pathway which activates during touch and defense response. In Arabidopsis, transcriptome analysis performed after touch treatment shows increased expression of genes which code for calcium binding proteins, defense response genes and Jasmonic acid/ ethylene responsive genes (Lee et al 2005, Pillai, S. E., & Patlavath, R 2015).

This indicates that plants recognise touch as threat and raise responses which alerts the plant for herbivore attack. In few plants, adaptation to mechanical stress provides tolerance to biotic and abiotic stress as well (Biddington 1986, Chehab 2009). As mechanical stress induces expression of defense response genes, touch stimuli is also being studied for development of environment friendly techniques for pest/ disease control in plants (Catherine Coutand, 2020). In present study, we have identified two touch responsive gene (TCH) orthologues in *C. cajan*, which were expressed at early time point after touch stimuli. Expression of the two *AtTCH* gene orthologue (*CcTCH1-1* and *CcTCH2-2*) were found to be upregulated at 30 minutes after touch treatment

3.3. Materials and methods

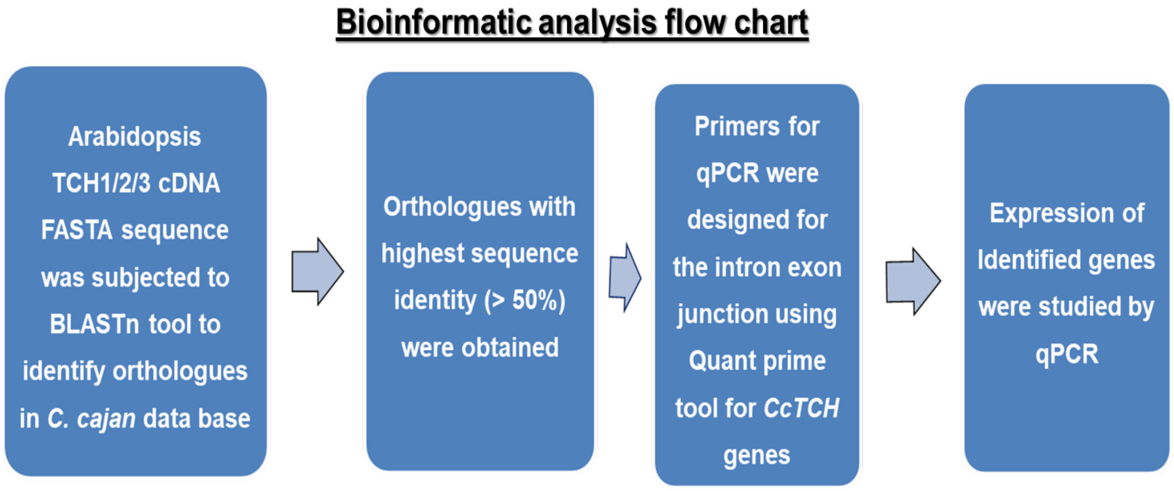


Figure 3.1: Flow chart of TCH gene identification and study of gene expression in C. cajan

3.3.1. Bioinformatic analysis of TCH genes:

The cDNA sequence of Arabidopsis TCH genes namely *AtTCH-1(CAM2), AtTCH-2* and *-AtTCH-3* were obtained from TAIR using accession no. AT2G41110.2, AT5G37770.1 and AT2G41100.1 respectively. For the three Arabidopsis TCH genes, the *C. cajan* orthologues were identified using the BLASTn algorithm at NCBI website (Altschul 1997). The *C. cajan* genes with highest identity (>50%) were further studied.

3.3.2. Multiple sequence alignment of Arabidopsis and *C. cajan* TCH genes:

We performed a comparative analysis of Arabidopsis TCH genes and their *C. cajan* orthologues using clustalW in EMBL site (https://www.ebi.ac.uk/Tools/msa/clustalo/). The percentage similarity matrix data was obtained from the same tool. Dendrogram for all three TCH genes were generated using same bioinformatic tool with Neighbor joining method without distance correction.

3.3.3. RT-PCR and Quantitative PCR analysis:

The expression of predicted TCH gene orthologues from *C. cajan* were studied using qPCR at thirty minutes after touch treatment. Primers were designed using Quant prime tool (Table 3.2). For this total RNA was extracted from control and touch treated plant tissue using TRIzol reagent (Thermo Fisher Scientific). Briefly the entire seedling was frozen in liquid nitrogen and crushed in TRIzol. For cDNA synthesis 1 µg of the total RNA was subjected to single strand synthesis using Oligo dT primer (Invitrogen). The cDNA was diluted ten times and used for qPCR analysis using SYBR green/ROX Master Mix (Thermo Fisher Scientific) on the 7900 HT sequence detection system (Applied Biosystem). *GAPDH* was used as internal control. The fold change in expression for the four *CcTCH* genes between untouched (control) and touched seedling was calculated using the $2^{-\Delta\Delta Ct}$ formula

(Livak and Schmittgen, 2001). Such three replicates were used for calculating the average fold change.

Table 3.1. List of primers used in for gene expression studies.

Name	**Primer sequence (5’->3’)**	**Reference**
CcTCH1-1F	CTTGGGCAGAACCCAACTGA	Designed in present work
CcTCH1-1R	AGCTCCTCCTCTGAATCGGT	Designed in present work
CcTCH1-2F	GAAGTGGATGCAGACGGGAA	Designed in present work
CcTCH1-2R	ACCCGGAATGCCTCTTTCAG	Designed in present work
CcTCH2-1F	GCGACGGCTACATTGACCTA	Designed in present work
CcTCH2-1R	CAGCCCGTTCTTGTCCAGAT	Designed in present work
CcTCH2-2F	CTCAAGGAGTTCGCCGACTT	Designed in present work
CcTCH2-2R	GGAGATGAGGCCGTTCTTGT	Designed in present work

3.3.4. Statistical analysis:

All the experiments were repeated in more than three independent biological replicates. For significance, all the data were analysed with Student’s *t*-test for independent means using Microsoft Excel software.

3.3.5. FASTA Sequences of TCH genes used for Bioinformatic Analysis

>Arabidopsis AtTCH1/CAM2

ATGGCGGATC AGCTCACAGA CGATCAGATC TCAGAATTCA AGGAAGCCTT
CAGCTTATTC GACAAGGATG GTGATGGTAT GCTTCATCCT CCCTTTCCCT
CTATCATCGT AGGTTGCATT ACCACAAAGG AGCTTGGTAC CGTGATGCGT
TCCCTCGGTC AAAACCCAAC CGAAGCTGAG CTTCAGGACA TGATCAACGA
AGTTGATGCG GATGGTAACG GAACCATTGA TTTCCCGGAG TTCTTGAACC
TAATGGCTAG GAAAATGAAG GACACTGACT CTGAGGAAGA ACTCAAGGAA
GCTTTCAGAG TTTTCGACAA AGACCAGAAC GGTTTCATCT CAGCTGCTGA

ATTGAGACAT GTGATGACTA ACCTCGGCGA GAAGCTTACT GATGAAGAAG

TTGATGAGAT GATTAAGGAA GCTGATGTTG ATGGTGATGG TCAGATCAAC

TACGAAGAGT TTGTGAAGGT TATGATGGCT AAGTGA

> *C. cajan*_05680 (*CcTCH1-1*)

ATGGCCGATCAACTCACTGACGAACAGATCTCCGAGTTCAAGGAGGCATTCAG

CCTCTTCGACAAGGACGGCGATGGTTGTATTACTACCAAGGAACTTGGGACCG

TGATGCGGTCACTTGGGCAGAACCCAACTGAGGCTGAGCTGCAGGACATGATA

AATGAGGTTGATGCTGATGGCAATGGCACCATTGATTTCCCAGAATTCCTGAA

TCTGATGGCACGCAAGATGAAAGACACCGATTCAGAGGAGGAGCTGAAGGAG

GCCTTCCGTGTGTTCGACAAGGATCAGAATGGTTTCATCTCTGCCGCCGAGCTG

CGCCACGTGATGACCAATCTTGGCGAGAAGCTGACCGACGAGGAAGTCGATG

AGATGATCCGGGAGGCTGATGTTGACGGTGATGGGCAGATCAACTATGAGGA

GTTTGTCAAAGTCATGATGGCCAAGTGA

> *C. cajan*_40611 (*CcTCH1-2*)

ATGGCCGATCAACTCACCGATGAACAGATCTCCGAGTTCAAGGAAGCCTTCAG

CTTGTTCGACAAGGACGGCGATGGTTGCATCACAACCAAGGAGCTTGGAACTG

TTATGCGCTCATTGGGGCAAAACCCAACTGAGGCAGAGCTCCAGGACATGATT

AATGAAGTGGATGCAGACGGGAATGGCACCATCGATTTCCCTGAGTTTCTAAA

CCTCATGGCCCGGAAGATGAAGGACACTGATTCTGAGGAAGAGCTGAAAGAG

GCATTCCGGGTTTTCGACAAGGACCAGAATGGGTTCATCTCTGCTGCTGAACT

CCGCCATGTGATGACCAACCTTGGGGAGAAGCTCACTGATGAAGAGGTTGATG

AAATGATTCGTGAGGCTGATGTTGATGGCGATGGCCAAATAAACTATGAGGAG

TTCGTTAAGGTGATGATGGCCAAGTGA

> **Arabidopsis *AtTCH2***

ATGTCATCGA AGAACGGAGT TGTTCGTAGC TGTTTAGGAT CAATGGACGA
CATCAAAAAA GTCTTCCAAC GATTCGACAA AAACGGCGAC GGGAAAATCT
CCGTCGACGA GCTCAAAGAA GTGATCCGCG CTCTCTCACC AACAGCATCA
CCAGAAGAAA CAGTAACGAT GATGAAACAA TTCGATCTAG ACGGTAACGG
ATTCATAGAT CTGGACGAAT TCGTCGCGCT TTTCCAAATC GGAATCGGAG
GAGGAGGTAA CAATCGAAAC GACGTAAGCG ATTTGAAAGA AGCGTTTGAG
TTATATGATT TGGATGGTAA TGGAAGGATC TCGGCGAAAG AGCTTCATTC
AGTGATGAAG AATTTGGGTG AGAAGTGCTC TGTGCAAGAT TGTAAGAAGA
TGATTAGTAA AGTTGATATT GATGGTGATG GTTGTGTTAA TTTTGATGAG
TTTAAGAAGA TGATGAGTAA TGGTGGTGGT GCTTGA

> *C. cajan*_19575 (*CcTCH2-1*)

ATGGACGAGGAGGTGCGCAAGATCTTCAGCAAGTTCGACAAGAACGGCGACG
GCAAGATCTCCAGCGCCGAGCTCAAGGACCTCATGGCGGCGCTGGGATCCAA
GACCACGGCGGAGGAGGTGCGCCGCATGATGGCGGAGCTGGACCAGAACGGC
GACGGCTACATTGACCTAAAGGAGTTCGGGGAGTTTCACTGCGGCGGCGGCGG
CGGCGACGGGAGGGAGCTCCGGGAGGCGTTCGAGCTGTACGATCTGGACAAG
AACGGGCTGATCTCGGCGAAGGAGCTGCATTCGGTGATGAGGAGGTTGGGGG
AGAAGTGCTCCCTCAGTGACTGCCGGAGGATGATCGGAAACGTCGACGCCGA
CGGCGATGGCAGCGTCAATTTCGAAGAGTTCAAGAAGATGATGACTCGCTCCT
AG

> *C. cajan*_05519 *(CcTCH2-2)*

ATGGACGACGAGGTGCGCCAGATCTTCAACAAGTTCGACAAGAACGGCGACG

GCAAGATCTCCATCGCCGAGCTCAAGGACATGCTCGTCACGCTCGGCTCCAAA

ACGACGAACGACGAGCTCAAACGCATGATGGAGGAGCTCGACCAAAACGGCG

ACGGTTTCATCGACCTCAAGGAGTTCGCCGACTTCCATTGCAATGACGCCGGA

AACGACGACTCCAAGGAGCTCCGCGACGCCTTCGATCTCTACGACGTCGACAA

GAACGGCCTCATCTCCGCCAAGGAGTTGCACGACGTGCTCCGGAGGCTCGGCG

AGAAGTGCTCCCTCAGCGACTGCCGCAAGATGATCAGCAACGTCGACGCCGAC

GGCGATGGCAACGTCAACTTCGAGGAGTTTAAGAAGATGATGGCTCGCTCTTA

G

> Arabidopsis *AtTCH3*

ATGGCGGATA AGCTCACTGA CGATCAGATT ACAGAATACA GGGAATCTTT

CAGGTTATTC GACAAGAATG GTGATGGTTC CATTACGAAA AAGGAGCTCG

GTACCATGAT GCGTTCAATC GGTGAAAAAC CGACAAAAGC TGATCTTCAG

GACTTGATGA ACGAAGCGGA TTTAGATGGT GATGGAACCA TCGATTTCCC

TGAGTTCTTG TGCGTAATGG CTAAGAATCA AGGTCATGAT CAAGCGCCGC

GTCACACTAA AAAAACAATG GCGGATAAGC TCACTGACGA TCAGATTACA

GAGTACAGGG AATCTTTCAG GTTATTCGAC AAGAATGGTG ATGGTTCCAT

TACGAAAAAG GAGCTCCGTA CCGTGATGTT TTCCCTCGGT AAAAACCGGA

CAAAAGCTGA TCTTCAGGAC ATGATGAACG AAGTGGATTT AGATGGTGAT

GGAACCATCG ATTTCCCTGA GTTCTTGTAC CTAATGGCTA AGAATCAAGG

TCATGATCAA GCGCCGCGTC ACACTAAAAA AACAATGGTG GATTATCAGC

TCACTGACGA TCAGATCTTA GAATTCAGGG AAGCCTTCCG CGTATTCGAC

AAGAATGGTG ATGGTTACAT TACCGTGAAT GAGCTCCGTA CTACTATGCG

CTCCCTTGGT GAAACCCAAA CAAAAGCTGA GCTCCAGGAC ATGATCAACG

AAGCGGATGC AGATGGTGAC GGAACCATCA GTTTCTCTGA GTTTGTGTGT

GTAATGACTG GTAAAATGAT TGACACTCAG TCTAAGAAAG AAACGTACAG

AGTTGTGAAT CAAGGTCAGG GTCAAGTGCA GCGTCACACT AGAAATGACA

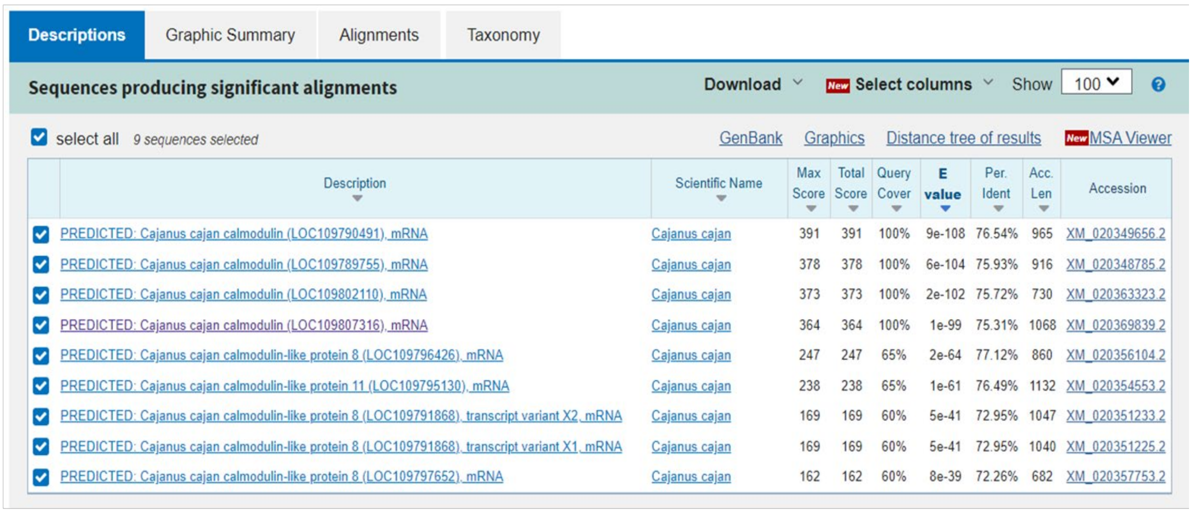
GAGCTGGTGG CACCAATTGG GAGAGGGACA TAGCGGTCGG GGTTGCCAGC

AATATCATCG CTTCGCCAAT TTCCGACTTC ATGAAAGATA GGTTTAAAGA

TTTGTTCGAA GCGCTGTTAT CTTGA

a

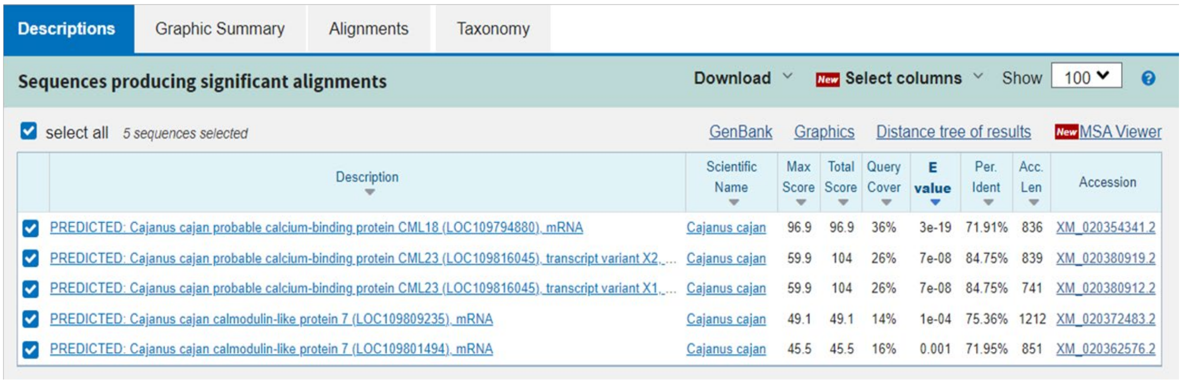
Blastn out put for *AtTCH1* gene



	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	PREDICTED: Cajanus cajan calmodulin (LOC109790491), mRNA	Cajanus cajan	391	391	100%	9e-108	76.54%	965	XM_020349656.2
☑	PREDICTED: Cajanus cajan calmodulin (LOC109789755), mRNA	Cajanus cajan	378	378	100%	6e-104	75.93%	916	XM_020348785.2
☑	PREDICTED: Cajanus cajan calmodulin (LOC109802110), mRNA	Cajanus cajan	373	373	100%	2e-102	75.72%	730	XM_020363323.2
☑	PREDICTED: Cajanus cajan calmodulin (LOC109807316), mRNA	Cajanus cajan	364	364	100%	1e-99	75.31%	1068	XM_020369839.2
☑	PREDICTED: Cajanus cajan calmodulin-like protein 8 (LOC109796426), mRNA	Cajanus cajan	247	247	65%	2e-64	77.12%	860	XM_020356104.2
☑	PREDICTED: Cajanus cajan calmodulin-like protein 11 (LOC109795130), mRNA	Cajanus cajan	238	238	65%	1e-61	76.49%	1132	XM_020354553.2
☑	PREDICTED: Cajanus cajan calmodulin-like protein 8 (LOC109791868), transcript variant X2, mRNA	Cajanus cajan	169	169	60%	5e-41	72.95%	1047	XM_020351233.2
☑	PREDICTED: Cajanus cajan calmodulin-like protein 8 (LOC109791868), transcript variant X1, mRNA	Cajanus cajan	169	169	60%	5e-41	72.95%	1040	XM_020351225.2
☑	PREDICTED: Cajanus cajan calmodulin-like protein 8 (LOC109797652), mRNA	Cajanus cajan	162	162	60%	8e-39	72.26%	682	XM_020357753.2

b

Blastn out put for *AtTCH2* gene



	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	PREDICTED: Cajanus cajan probable calcium-binding protein CML18 (LOC109794880), mRNA	Cajanus cajan	96.9	96.9	36%	3e-19	71.91%	836	XM_020354341.2
☑	PREDICTED: Cajanus cajan probable calcium-binding protein CML23 (LOC109816045), transcript variant X2,...	Cajanus cajan	59.9	104	26%	7e-08	84.75%	839	XM_020380919.2
☑	PREDICTED: Cajanus cajan probable calcium-binding protein CML23 (LOC109816045), transcript variant X1,...	Cajanus cajan	59.9	104	26%	7e-08	84.75%	741	XM_020380912.2
☑	PREDICTED: Cajanus cajan calmodulin-like protein 7 (LOC109809235), mRNA	Cajanus cajan	49.1	49.1	14%	1e-04	75.36%	1212	XM_020372483.2
☑	PREDICTED: Cajanus cajan calmodulin-like protein 7 (LOC109801494), mRNA	Cajanus cajan	45.5	45.5	16%	0.001	71.95%	851	XM_020362576.2

***Figure 3.2**: For the three Arabidopsis TCH genes, the C. cajan orthologues were identified using the BLASTn algorithm at NCBI website. (a) shows the output for AtTCH1sequence search and (b) shows the output for AtTCH2. For AtTCH3 no close orthologue with more than 50% identity were found.*

3.4 Results:

3.4.1. Identification of Arabidopsis TCH gene orthologues in *C. cajan*

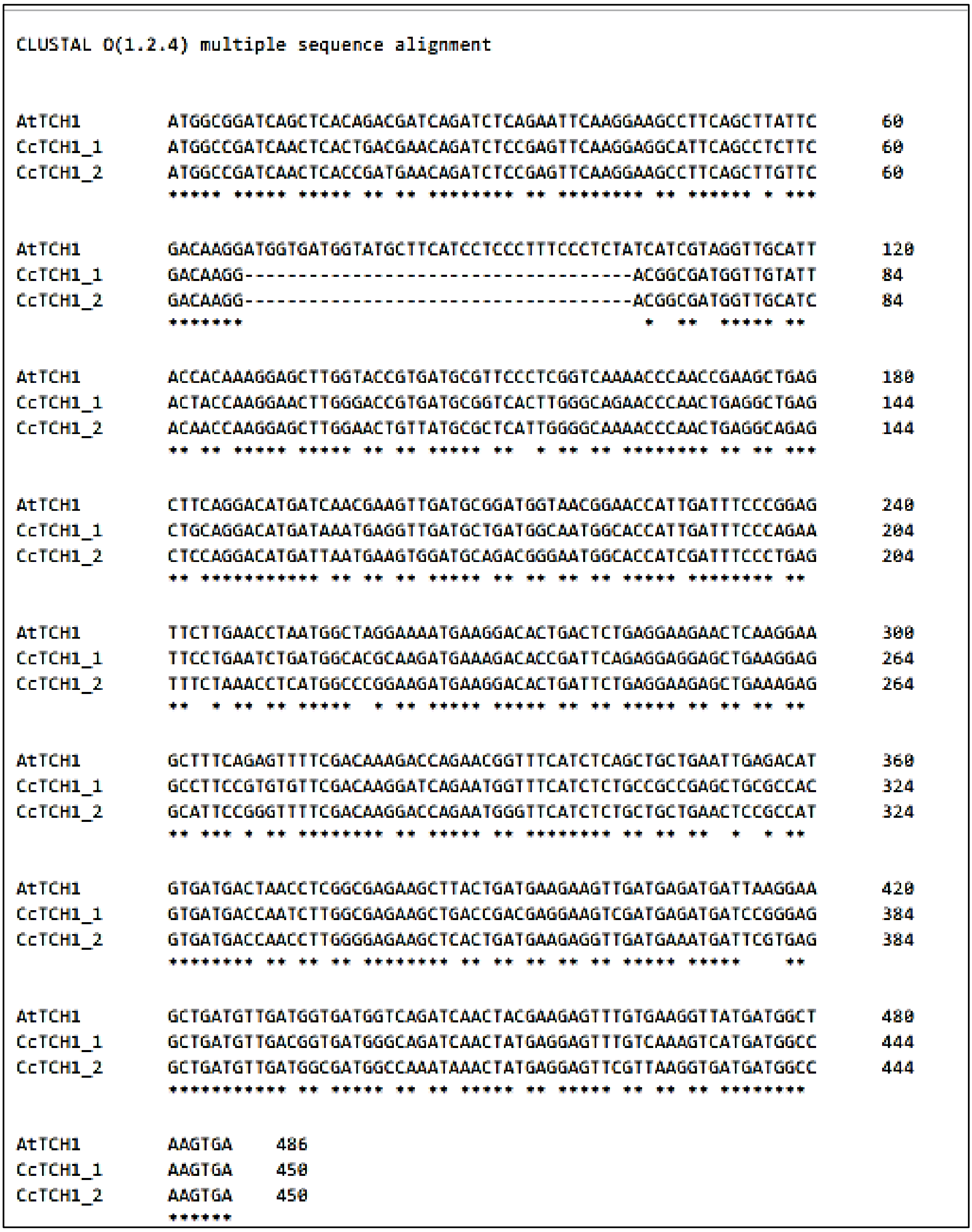
In Arabidopsis, Braam and her research group have reported presence of touch responsive genes which are expressed after touch treatment, namely *AtTCH1* (calmodulin;CaM), *AtTCH2* and *AtTCH3* (calmodulin like; CML). We made an attempt to identify these TCH genes orthologues in *C. cajan* using bioinformatic analysis. In order to identify *C. cajan* orthologues, FASTA sequence of the Arabidopsis TCH-1, -2, and -3 were subjected to analysis using BLASTn tool in NCBI data base. The *C. cajan* genes with highest identity (>50%) were identified in present study (**Table 3.1**). *CcTCH1-1* and *CcTCH1-2* were identified as putative orthologues for *AtTCH1* gene while *CcTCH2-1* and *CcTCH2-2* were identified as the putative orthologues for *AtTCH2* gene. For *AtTCH3*, no orthologues were obtained with more than 50% identity in our analysis. These putative orthologues were studied for gene expression upon touch treatment in *C. cajan*.

Table 3.2: Details of the predicted *C. cajan* TCH gene orthologs compared to Arabidopsis.

Arabidopsis TCH genes	**Arabidopsis Gene ID (TAIR)**	**Locus ID of *C. cajan* (NCBI)**	**Name given in present study**	**Identity**	**e-value (blastx)**	**Predicted Domain (NCBI)**
AtTCH 1 (CAM2)	AT2G41110.2	LOC109790491	*CcTCH1-1*	91.3	2.41 e-98	calmodulin
		LOC109807316	*CcTCH1-2*	91.3	2.41e-98	calmodulin
AtTCH 2	AT5G37770.1	LOC109816045	*CcTCH2-1*	56.0	6.23e-47	CML-23
		LOC109794880	*CcTCH2-2*	51.8	2.56e-46	CML-18

3.4.2. DNA sequence comparison of TCH orthologues:

The TCH gene and their *C. cajan* orthologues were submitted in clustal-W at EMBL site for sequence alignment. The alignment for both the TCH1 orthologues shows a 36-bps length of gap at the 5' end (Figure 3.3). *CcTCH1-1* and *CcTCH1-2* shows 80% and 81% sequence identity with the Arabidopsis TCH1 gene respectively (Figure 3.5a). The alignment for both the TCH2 orthologues shows three gaps at the 5' end, 206th position and at 3'end (Figure 3.3). *CcTCH2-1* and *CcTCH2-2* shows 60% and 56% sequence identity with the Arabidopsis TCH2 gene respectively (Figure 3.5a). A dendrogram was prepared for all the TCH genes studied here. It showed that TCH1, -2 and -3 form three different cluster. The AtTCH3 gene sequence is quite distinct from the other Arabidopsis TCH genes (Figure 3.6).



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CLUSTAL O(1.2.4) multiple sequence alignment


AtTCH1          ATGGCGGATCAGCTCACAGACGATCAGATCTCAGAATTCAAGGAAGCCTTCAGCTTATTC      60
CcTCH1_1        ATGGCCGATCAACTCACTGACGAACAGATCTCCGAGTTCAAGGAGGCATTCAGCCTCTTC      60
CcTCH1_2        ATGGCCGATCAACTCACCGATGAACAGATCTCCGAGTTCAAGGAAGCCTTCAGCTTGTTC      60
                ***** ***** ***** ** ** ******** ** ******** ** ****** * ***

AtTCH1          GACAAGGATGGTGATGGTATGCTTCATCCTCCCTTTCCCTCTATCATCGTAGGTTGCATT      120
CcTCH1_1        GACAAGG------------------------------------ACGGCGATGGTTGTATT      84
CcTCH1_2        GACAAGG------------------------------------ACGGCGATGGTTGCATC      84
                *******                                     *  **  ***** **

AtTCH1          ACCACAAAGGAGCTTGGTACCGTGATGCGTTCCCTCGGTCAAAACCCAACCGAAGCTGAG      180
CcTCH1_1        ACTACCAAGGAACTTGGGACCGTGATGCGGTCACTTGGGCAGAACCCAACTGAGGCTGAG      144
CcTCH1_2        ACAACCAAGGAGCTTGGAACTGTTATGCGCTCATTGGGGCAAAACCCAACTGAGGCAGAG      144
                ** ** ***** ***** ** ** ***** **  * ** ** ******** ** ** ***

AtTCH1          CTTCAGGACATGATCAACGAAGTTGATGCGGATGGTAACGGAACCATTGATTTCCCGGAG      240
CcTCH1_1        CTGCAGGACATGATAAATGAGGTTGATGCTGATGGCAATGGCACCATTGATTTCCCAGAA      204
CcTCH1_2        CTCCAGGACATGATTAATGAAGTGGATGCAGACGGGAATGGCACCATCGATTTCCCTGAG      204
                ** *********** ** ** ** ***** ** ** ** ** ***** ******** **

AtTCH1          TTCTTGAACCTAATGGCTAGGAAAATGAAGGACACTGACTCTGAGGAAGAACTCAAGGAA      300
CcTCH1_1        TTCCTGAATCTGATGGCACGCAAGATGAAAGACACCGATTCAGAGGAGGAGCTGAAGGAG      264
CcTCH1_2        TTTCTAAACCTCATGGCCCGGAAGATGAAGGACACTGATTCTGAGGAAGAGCTGAAAGAG      264
                **  * ** ** *****  * ** ***** ***** ** ** ***** ** ** ** **

AtTCH1          GCTTTCAGAGTTTTCGACAAAGACCAGAACGGTTTCATCTCAGCTGCTGAATTGAGACAT      360
CcTCH1_1        GCCTTCCGTGTGTTCGACAAGGATCAGAATGGTTTCATCTCTGCCGCCGAGCTGCGCCAC      324
CcTCH1_2        GCATTCCGGGTTTTCGACAAGGACCAGAATGGGTTCATCTCTGCTGCTGAACTCCGCCAT      324
                ** *** * ** ******** ** ***** ** ******** ** ** **  *  * **

AtTCH1          GTGATGACTAACCTCGGCGAGAAGCTTACTGATGAAGAAGTTGATGAGATGATTAAGGAA      420
CcTCH1_1        GTGATGACCAATCTTGGCGAGAAGCTGACCGACGAGGAAGTCGATGAGATGATCCGGGAG      384
CcTCH1_2        GTGATGACCAACCTTGGGGAGAAGCTCACTGATGAAGAGGTTGATGAAATGATTCGTGAG      384
                ******** ** ** ** ******** ** ** ** ** ** ***** *****    **

AtTCH1          GCTGATGTTGATGGTGATGGTCAGATCAACTACGAAGAGTTTGTGAAGGTTATGATGGCT      480
CcTCH1_1        GCTGATGTTGACGGTGATGGGCAGATCAACTATGAGGAGTTTGTCAAAGTCATGATGGCC      444
CcTCH1_2        GCTGATGTTGATGGCGATGGCCAAATAAACTATGAGGAGTTCGTTAAGGTGATGATGGCC      444
                *********** ** ***** ** ** ***** ** ***** ** ** ** ********

AtTCH1          AAGTGA    486
CcTCH1_1        AAGTGA    450
CcTCH1_2        AAGTGA    450
                ******
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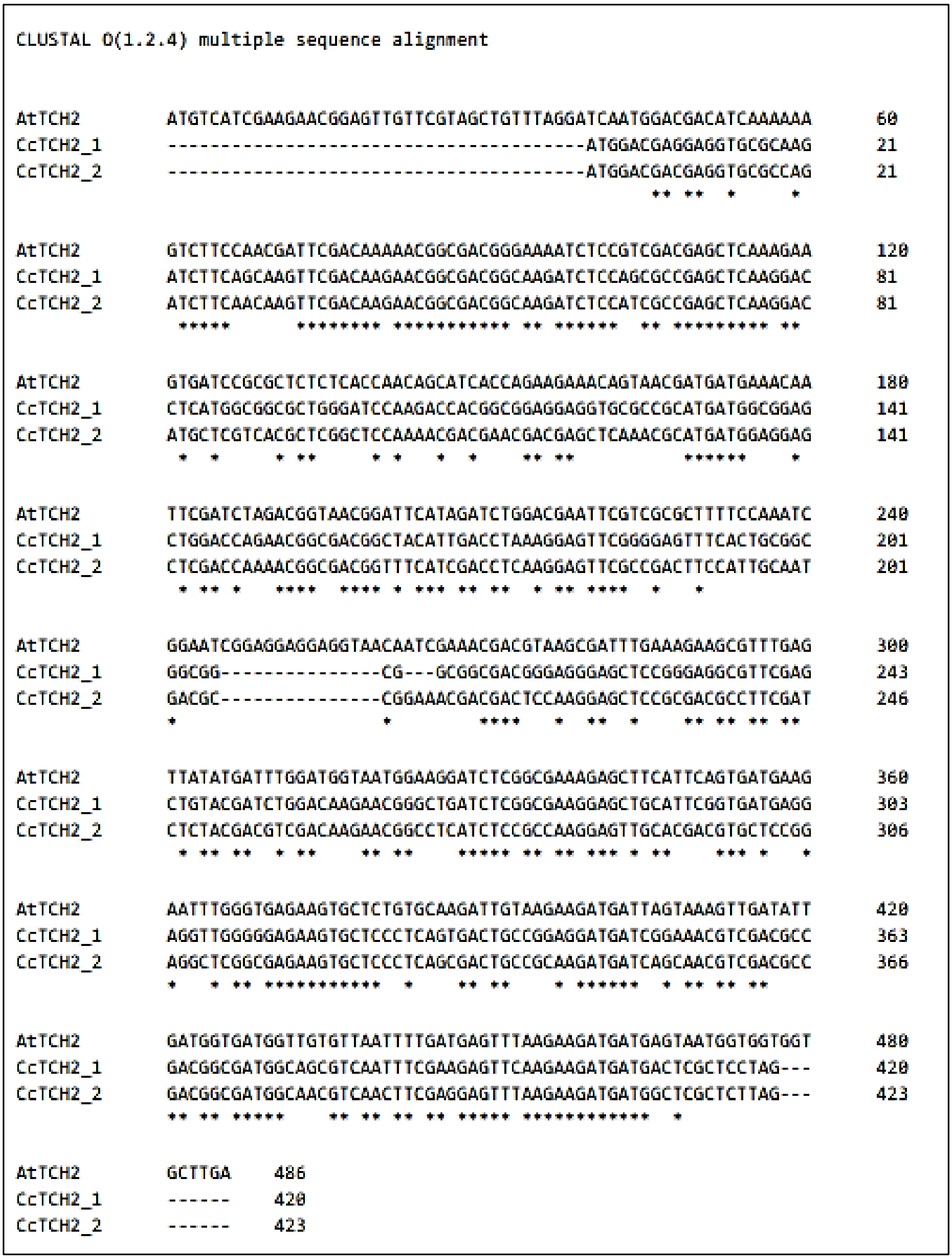
Figure 3.3: Multiple Sequence Alignment of Arabidopsis AtTCH1 with C. cajan orthologues: *The sequence alignment data shows presence of a gap after 67th base in the cDNA sequence. Key: * indicates identical conserved regions.*



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CLUSTAL O(1.2.4) multiple sequence alignment


AtTCH2        ATGTCATCGAAGAACGGAGTTGTTCGTAGCTGTTTAGGATCAATGGACGACATCAAAAAA      60
CcTCH2_1      ---------------------------------------ATGGACGAGGAGGTGCGCAAG      21
CcTCH2_2      ---------------------------------------ATGGACGACGAGGTGCGCCAG      21
                                                           ** **  *     *

AtTCH2        GTCTTCCAACGATTCGACAAAAACGGCGACGGGAAAATCTCCGTCGACGAGCTCAAAGAA      120
CcTCH2_1      ATCTTCAGCAAGTTCGACAAGAACGGCGACGGCAAGATCTCCAGCGCCGAGCTCAAGGAC      81
CcTCH2_2      ATCTTCAACAAGTTCGACAAGAACGGCGACGGCAAGATCTCCATCGCCGAGCTCAAGGAC      81
               *****      ******** *********** ** ******  ** ********* **

AtTCH2        GTGATCCGCGCTCTCTCACCAACAGCATCACCAGAAGAAACAGTAACGATGATGAAACAA      180
CcTCH2_1      CTCATGGCGGCGCTGGGATCCAAGACCACGGCGGAGGAGGTGCGCCGCATGATGGCGGAG      141
CcTCH2_2      ATGCTCGTCACGCTCGGCTCCAAAACGACGAACGACGAGCTCAAACGCATGATGGAGGAG      141
               *  *     * **     * *   *  *    ** **          ******    *

AtTCH2        TTCGATCTAGACGGTAACGGATTCATAGATCTGGACGAATTCGTCGCGCTTTTCCAAATC      240
CcTCH2_1      CTGGACCAGAACGGCGACGGCTACATTGACCTAAAGGAGTTCGGGGAGTTTCACTGCGGC      201
CcTCH2_2      CTCGACCAAAACGGCGACGGTTTCATCGACCTCAAGGAGTTCGCCGACTTCCATTGCAAT      201
               * ** *   ****  **** * *** ** **  * ** ****  *   *

AtTCH2        GGAATCGGAGGAGGAGGTAACAATCGAAACGACGTAAGCGATTTGAAAGAAGCGTTTGAG      300
CcTCH2_1      GGCGG---------------CG---GCGGCGACGGGAGGGAGCTCCGGGAGGCGTTCGAG      243
CcTCH2_2      GACGC---------------CGGAAACGACGACTCCAAGGAGCTCCGCGACGCCTTCGAT      246
              *                   *        ****   *  **  *    ** ** ** **

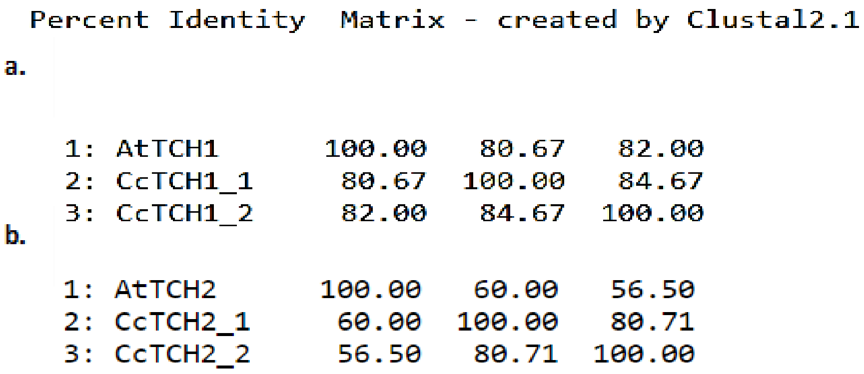
AtTCH2        TTATATGATTTGGATGGTAATGGAAGGATCTCGGCGAAAGAGCTTCATTCAGTGATGAAG      360
CcTCH2_1      CTGTACGATCTGGACAAGAACGGGCTGATCTCGGCGAAGGAGCTGCATTCGGTGATGAGG      303
CcTCH2_2      CTCTACGACGTCGACAAGAACGGCCTCATCTCCGCCAAGGAGTTGCACGACGTGCTCCGG      306
               * ** **  * **    ** **    ***** ** ** *** * **    *** *   *

AtTCH2        AATTTGGGTGAGAAGTGCTCTGTGCAAGATTGTAAGAAGATGATTAGTAAAGTTGATATT      420
CcTCH2_1      AGGTTGGGGGAGAAGTGCTCCCTCAGTGACTGCCGGAGGATGATCGGAAACGTCGACGCC      363
CcTCH2_2      AGGCTCGGCGAGAAGTGCTCCCTCAGCGACTGCCGCAAGATGATCAGCAACGTCGACGCC      366
              *   * ** ***********  *    ** **    * ******  * ** ** **

AtTCH2        GATGGTGATGGTTGTGTTAATTTTGATGAGTTTAAGAAGATGATGAGTAATGGTGGTGGT      480
CcTCH2_1      GACGGCGATGGCAGCGTCAATTTCGAAGAGTTCAAGAAGATGATGACTCGCTCCTAG---      420
CcTCH2_2      GACGGCGATGGCAACGTCAACTTCGAGGAGTTTAAGAAGATGATGGCTCGCTCTTAG---      423
              ** ** *****    ** ** ** ** ***** ************  *

AtTCH2        GCTTGA    486
CcTCH2_1      ------    420
CcTCH2_2      ------    423
```

Figure 3.4: Multiple Sequence Alignment of Arabidopsis AtTCH2 with C. cajan orthologues: *The sequence alignment data shows presence of 3 large gaps in the cDNA sequence. Key: * indicates identical conserved regions*



```
 Percent Identity  Matrix - created by Clustal2.1
a.

   1: AtTCH1      100.00   80.67   82.00
   2: CcTCH1_1     80.67  100.00   84.67
   3: CcTCH1_2     82.00   84.67  100.00
b.

   1: AtTCH2      100.00   60.00   56.50
   2: CcTCH2_1     60.00  100.00   80.71
   3: CcTCH2_2     56.50   80.71  100.00
```

Figure 3.5: Percent Identity Matrix for multiple sequence alignment of TCH gene orthologues.

a and b represent the percent identity for TCH1 and TCH2 gene respectively. The DNA FASTA sequence was submitted to clustalW analysis to obtain the above information.

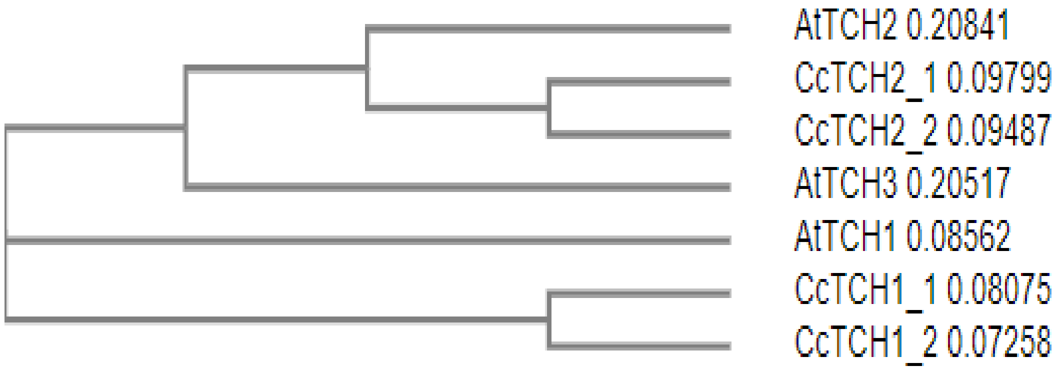


Figure 3.6: Dendrogram representing the sequence relatedness of AtTCH-1, -2 and -3 and their C. cajan orthologues. *Its distinctly clear that the TCH3 sequence is very different from that of TCH-1 and -2. The dendrogram was constructed using Neighbour joining without distance correction algorithm.*

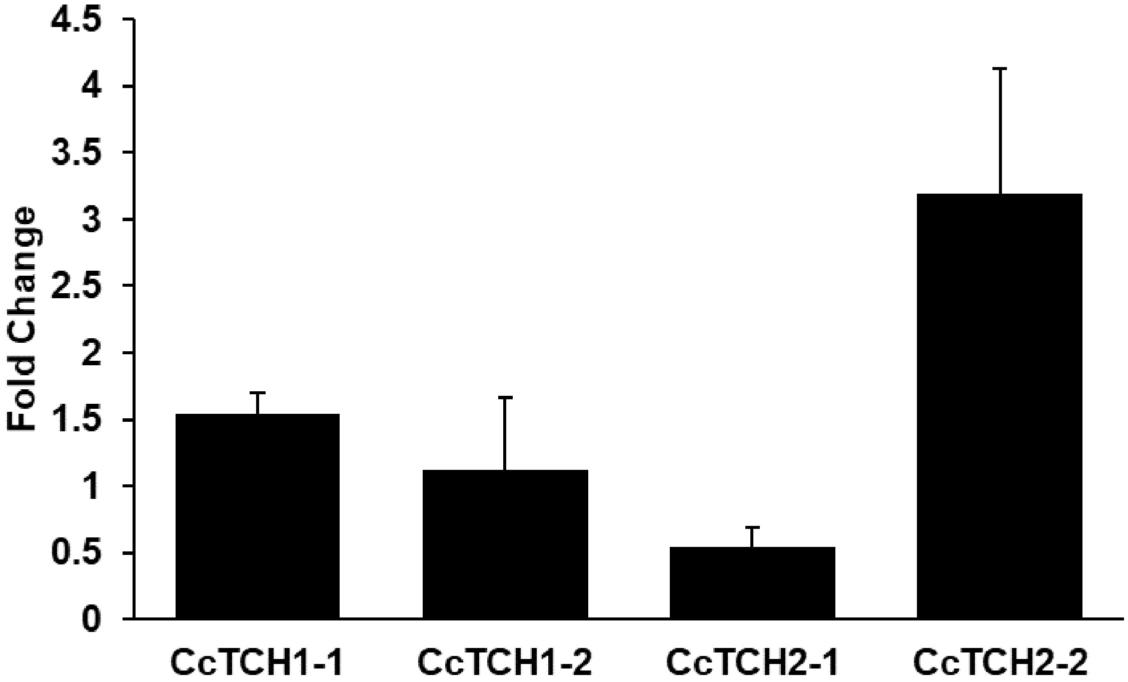


Figure 3.7: Effect of touch stimuli on expression of TCH gene orthologues in C. cajan

The expression of the above identified TCH orthologues was studied by quantitative PCR at 30 and 60 minutes in leaves. At 30 minutes post treatment, leaves were collected and processed for qRT-PCR. The relative fold change was calculated over untouched leaves (for wound treatment). GAPDH was used as internal control. The graph represents the mean of three experimental replicates and error bar represents standard deviation.

3.4.3. Expression of TCH2 orthologues (*CcTCH2*) is upregulated upon touch treatment:

The gene expression of the above identified putative TCH gene orthologues was studied by quantitative PCR at 30 and 60 minutes in leaves. At 30 minutes, expression of *CcTCH1-1* was upregulated by 1.5-fold over the control and the levels of *CcTCH1-2* remain unchanged after touch treatment (Figure 3.7). In case of the TCH-2 orthologues, expression of CcTCH2-1 was downregulated (0.5-fold) while that of CcTCH2-2 was significantly upregulated by 3-fold. However, at 60 minutes after touch treatment the expression levels of all the TCH orthologues were back to normal, the Ct values of touch treated and untouched plants were similar

3.5. DISCUSSION:

Cytosolic calcium (Ca $^{2+}$) levels are altered upon mechanical perturbations like touch and wounding in plants (Cosgrove and Hedrich 1991). Ca^{2+} mediated intracellular communication is generally facilitated by calcium sensors; calmodulin (CaM) and calmodulin like-molecules (CML). Gene expression studies in Arabidopsis shows that expression of many CaM and CML molecules is induced within 30 min of touch stimulation (Braam and Davis 1990; Lee et al 2005). In Arabidopsis, Braam and co-workers have extensively worked on few of the touch induced CaM and CML also as known as TCH genes (Braam and Davis 1990). In our work we have attempted to identify *C. cajan* counterparts of three TCH genes namely *AtTCH1* (calmodulin; CaM2), *AtTCH2* (CML24) and *AtTCH3* (CML12).

Nucleic acid and proteins are modified products of evolution. During evolution DNA sequences progressively accumulate mutations, traces of evolution may still remain in certain regions of the sequences to allow identification of the common ancestry. Thus, we performed a comparative analysis of Arabidopsis TCH genes and their *C. cajan* orthologues. The *CcTCH1-1* and *CcTCH2-2* cDNA sequence showed more similarity with *AtTCH1* gene indicating that the gene has fewer mutations. The 36bps gap at N'-terminal end could have resulted due to deletion mutation in *C. cajan* during evolution. The *CcTCH2-1* and *CcTCH2-2* cDNA sequence showed multiple gaps and less similarity with *AtTCH2.* The CcTCH2 genes have evolved multiple deletions and point mutation specific to the genera.

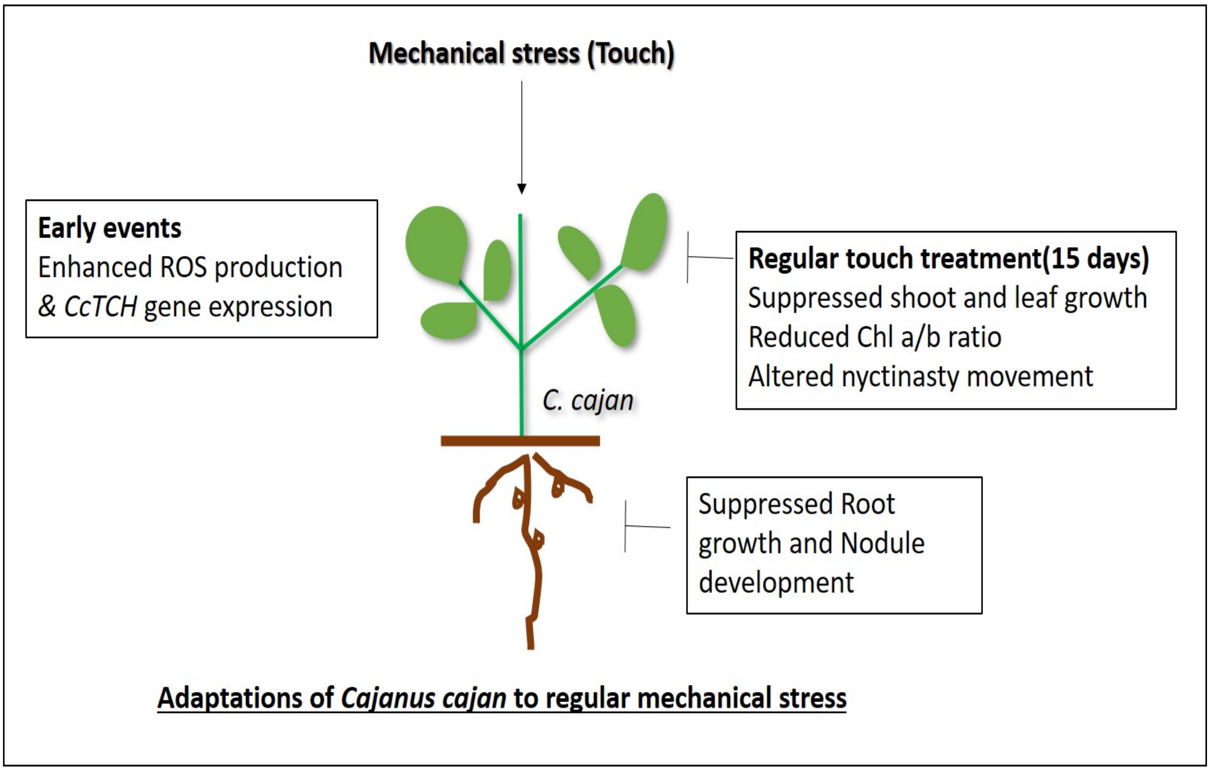


Figure 3.8: Adaptations of C. cajan to regular mechanical stress.

Touch stimuli enhanced ROS production and expression of two touch responsive gene (CcTCH) orthologues in C. cajan at an early time point. Regular touch treatment for 15 days suppresses overall plant growth, enhances lignin deposition, and reduces chlorophyll a/b ratio (Chapter 2). Also, the properties common to legume family like nyctinasty movement of leaves and root nodule development, were negatively affected by regular touch treatment.

The expression of two TCH orthologues (*CcTCH1-1* and *CcTCH2-2*) were upregulated at 30 minutes. This indicates presence of a calmodulin like molecules in *C. cajan* similar to that in Arabidopsis. The levels of *CcTCH1-1* and *CcTCH2-2* were back to normal at 60 minutes when compared to controls. This indicated that the expression of these genes is tightly regulated in *C. cajan* to avoid unnecessary expenditure of energy in raising a stress response. How does plant perceive touch- the exact mechanism is not yet clear? However, it is proposed that touching plant increases levels of ROS which in turn may also lead to activation of calcium channels in plants (Moris et al 2004). This triggers expression of touch responsive gene which are calcium binding proteins like TCH genes.

3.6. CONCLUSIONS:

Gene expression studies gives us an insight on presence of a conserved mechanisms for touch mediated response in *C. cajan*. The touch induced phenotypes and genes identified in present study can be used as marker for mechanical stress in *C. cajan* for future studies.

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