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ORIGINAL PAPER

A novel mutation in *TFL1* homolog affecting determinacy in cowpea (*Vigna unguiculata*)

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Abstract Mutations in the widely conserved *Arabidopsis Terminal Flower 1* (*TFL1*) gene and its homologs have been demonstrated to result in determinacy across genera, the knowledge of which is lacking in cowpea. Understanding the molecular events leading to determinacy of apical meristems could hasten development of cowpea varieties with suitable ideotypes. Isolation and characterization of a novel mutation in cowpea *TFL1* homolog (*VuTFL1*) affecting determinacy is reported here for the first time. Cowpea *TFL1* homolog was amplified using primers designed based on conserved sequences in related genera and sequence variation was analysed in three gamma ray-induced determinate mutants, their indeterminate parent "EC394763" and two indeterminate varieties. The analyses of sequence variation exposed a novel SNP distinguishing the determinate mutants from the indeterminate types. The non-synonymous point mutation in exon 4 at position 1,176 resulted from transversion of cytosine (C) to adenine (A) leading to an amino acid change (Pro-136 to His) in determinate mutants. The effect of the mutation on protein function and stability was predicted to be detrimental using different bioinformatics/computational tools. The functionally significant novel substitution mutation is hypothesized to affect determinacy in the cowpea mutants. Development of suitable regeneration protocols in this hitherto recalcitrant crop and subsequent complementation assay in mutants or over-expressing assay in parents could decisively conclude the role of the SNP in regulating determinacy in these cowpea mutants.

Keywords Determinate growth habit · Terminal flower · Point mutation · *VuTFL1* · Cowpea · *Vigna unguiculata*

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Introduction

Cowpea (*Vigna unguiculata* (L.) Walp) is a globally important protein-rich legume crop cultivated widely in the arid and semi-arid regions of the world. Bestowed with the ability to tide over abiotic stresses like drought and heat, cowpea assumes significance in the present context of climate change. Cowpea exhibits genetic variability for growth habit types that are generally classified as determinate or indeterminate. A plant exhibiting indeterminate growth habit will have a terminal shoot meristem that remains in a vegetative state throughout the production of vegetative and reproductive structures; whereas, in plants showing determinate growth habit, the terminal meristem will switch from a vegetative to a reproductive state, thus, producing a terminal flower. Determinate growth habit, supposedly a domesticated trait (Kwak et al. 2012), aims at decreasing plant biomass and optimizes photosynthates allocation between vegetative and reproductive growth (Cober and Tanner 1995). Determinacy also averts inter-twining of adjacent plants facilitating easy execution of intercultural operations like weeding, spraying agro-chemicals and harvesting. With the added advantage of synchronous maturity and amenability to mechanical harvesting, restructuring of traditional indeterminate cowpea varieties towards determinacy would enhance its adaptability and climate change

resilience potential. Thus, modern breeding efforts are streamlined at development of cowpea cultivars with determinacy trait that has been preferred in several crop species (Pnueli et al. 1998; Kelly 2001; Boote et al. 2003).

The architecture of a plant is specified through the activities of indeterminate and determinate meristems and the sum of these events sharply impact plant growth habit, productivity and crop management. Understanding of the molecular functions that control the phase transition from vegetative to reproductive growth has emanated from various studies on the apical meristem identity in many higher plants (Pidkowich et al. 1999). The CENTRORADIALIS/TERMINAL FLOWER 1/SELF-PRUNING (CETS) gene family sharing homology with phosphatidylethanolamine binding protein (PEBP) genes prominently controls timing and location of the developmental transition from indeterminate to determinate growth, with different family members balancing the activities of others through antagonistic functions. The CETS member FLOWERING LOCUS T (*FT*) of *Arabidopsis* and related genes are important in promoting the transition to determinate growth while *TERMINAL FLOWER 1* (*TFL1*) and its homologs oppose this activity by maintaining meristems in an indeterminate state (McGarry and Ayre 2012). In *Arabidopsis*, the floral meristem fate is decided by two opposing pathways. *FT* interacts with FLOWERING LOCUS D (*FD*) to form a heterodimer that binds to the promoter of APETALA 1 (*AP1*) to activate flowering initiation (Wigge et al. 2005). Antagonistically, *TFL1* acts as a repressor for floral initiation and maintains the inflorescence meristem through suppression of the expression of *AP1* and LEAFY (*LFY*) flowering genes (Boss et al. 2004).

In contrast to proteins such as *CO*, *FLC* or *LFY*, which encode transcription factors (Parcy et al. 1998; Michaels and Amasino 1999; Sheldon et al. 1999; Samach et al. 2000), *FT* and *TFL1* encode small proteins of about 175 amino acids. As a member of PEBP family, *CEN/TFL1* like genes, are found to be involved in signalling transduction pathway (Banfield and Brady 2000; Pnueli et al. 2001). *TFL1* therefore controls plant architecture by determining where and when flowers are made by delaying the switch from vegetative phase to flowering.

Mutations in *CEN/TFL1*-like genes have been shown to result in the inflorescence meristem being converted into a terminal flower (Shannon and Meeks-Wagner 1991; Bradley et al. 1996). Gain-of-function studies have revealed that protein sequence rather than expression pattern, largely determine the different functions of *TFL1* and *FT* (Ratcliffe et al. 1998; Kardailsky et al. 1999; Kobayashi et al. 1999). Even a single base substitution has the potential to interchangeably convert the activities of *FT* to *TFL1* and vice versa (Hanzawa et al. 2005).

Analyses of *CEN/TFL1*-like genes from diverse species including monocots and eudicots have revealed that they are highly conserved and generally contain three introns and four exons (Tahery et al. 2011). Key amino acids responsible for the functional divergence between *TFL1* and *FT* proteins with high sequence similarity have been identified and in specific Tyr85His in *FT* and His88Tyr in *TFL1* were found to be important residues for antagonistic functions (Hanzawa et al. 2005). The most substantial difference between *FT* and *TFL1* has been identified to be in the external loop (residues 128–145), which is critical for *FT* versus *TFL1* activity in vivo. The 17 amino acid long segment B of the fourth exon of *TFL1* is not only very different between *FT* and *TFL1*, but also evolves very rapidly between *TFL1* orthologs (Ahn et al. 2006). It has been observed that while exon 4 offers great plasticity for evolution of determinate plant types from indeterminate plant types, the exons one to three are generally conserved. Isolation of putative orthologs and their molecular characterization involving RNA and/or protein expression patterns provides insights into the conservation and diversification of gene function. Even though *TFL* homologs have been identified and reported in a number of crops, till date no counterpart has been identified in cowpea. Hence, the present investigation was carried out with a view to isolating and characterizing cowpea *TFL1* homolog along with perceiving its evolutionary divergence. A mutagenesis experiment was also conceived to induce determinacy in cowpea and to utilize the determinate mutant(s) to discern the possible molecular event leading to determinate growth habit.

Materials and methods

Plant material

A high-yielding exotic cowpea germplasm "EC394763" with indeterminate growth habit was mutagenized with 220 Gy gamma rays. Around 2,000 seeds (200 g) were irradiated and M_1 generation was raised at the Station's Experimental Field at Trombay, India, during rainy season of 2010. Fourteen hundred and eighty-three M_1 plants were harvested individually and advanced to M_2 as plant to row progenies during rainy season of 2011. Morphological mutants were identified in M_2 generation and were harvested individually. The true breeding behaviour of the mutants was studied by forwarding up to M_6 generations. Three determinate M_6 mutants ("TCM418", "TCM420" and "TCM440") and the parent along with five other mutants were grown in a randomized block design with three replications. Morphological data with respect to plant height, number of nodes, number of branches, mean branch length (sum of length of all branches/number of branches), mean internodal length (length of central axis/number of internodes), number of leaves, number of pod clusters

(peduncles bearing pods), pod length, seeds per pod, seed yield per plant, days to flower and days to maturity were recorded on five random plants per replication and the mean was used for performing ANOVA. Seeds of the three determinate M_6 mutants, parent and two indeterminate varieties ("V-130" and "V-240") were used for DNA extraction.

Primer design

For amplification of the putative growth habit gene in cowpea, five primer pairs (Online Resource 1) were synthesized corresponding to conserved domains of varying intronic and exonic regions identified from the alignment of published *CEN/TFL1* homologs. The monomorphic band amplified by the primer pair TFL-1F (5′-CCTGGCCCTAGTGATCC TTA-3′) and TFL-1R (5′-GCGTCTTCTTGCAGCGGTT-3′) was sequenced. The end sequences of the complete coding region of *Phaseolus vulgaris TFLly* (*PvTFLly*) homolog having maximum similarity with the amplified sequence (based on BLAST alignment) was further used to design the end primers TFL-6F (5′-ATGGCAAGAATGCCTTT AGAACC-3′) and TFL-6R (5′-CTAGCGTCTTCTTGCAG CTGTTT-3′) for amplifying the cowpea homolog. The NCBI primer-BLAST software Primer3 (Ye et al. 2012) was used for designing the primers.

DNA extraction, PCR conditions, cloning and sequencing

Genomic DNA of M_6 mutants, parents and the two indeterminate varieties was extracted according to the protocol of Dellaporta et al. (1983) with minor modifications as described by Dhanasekar et al. (2010) except that overnight soaked seeds and not leaves were crushed directly in extraction buffer without use of liquid nitrogen. NanoDrop 2000 (Thermo Scientific, US) quantified DNA was PCR amplified in an Eppendorf Master-Cycler gradient (Eppendorf Netheler-Hinz GMBH, Hamburg), employing 50 ng of template DNA, 0.5 U of *Taq* DNA polymerase (Bangalore Genei Ltd.), 0.4 mM of dNTPs, 10 pmol of each forward and reverse primers in a 1× PCR buffer [10 mM Tris (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01 % gelatin] in a 25 µl reaction volume. The thermal profiles for amplification differed with respect to the primer pairs: TFL-1F/R and TFL-6F/R. For former, an initial denaturation step (10 min at 94 °C) followed by 30 cycles of 94 °C for 35 s, 60 °C for 35 s and 72 °C for 45 s and terminating with a final extension at 72 °C for 7 min was used, whereas for latter an initial denaturation step (4 min at 94 °C) followed by 30 cycles of 94 °C for 30 s, 52 °C for 1 min and 72 °C for 2 min and with a final extension at 72 °C for 10 min was used. PCR amplification products were size-separated by standard horizontal electrophoresis in 2 % agarose (Sigma, St. Louis, USA) gels at 75 V. The PCR products were excised and purified using GenElute™ Gel Extraction Kit (Sigma-Aldrich, US). The TFL-1 F/R amplified PCR products were used directly for sequencing, while the TFL-6 F/R amplified PCR products were ligated directly into *pTZ57R* vector (InsTAclone™ PCR Product Cloning Kit, Fermentas, Lithuania) according to the manufacturer's instructions for transformation of *Escherichia coli* DH5α cells. The transformed colonies containing the cloned fragment were identified following standard blue-white screening and colony PCR. Overnight grown cultures of positive colonies were used for plasmid DNA isolation using GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, USA) following manufacturer's instructions. The plasmid DNA in duplicates was then used for Sanger sequencing (Applied Biosystems, USA).

Sequence analysis

The complete sequences of the putative cowpea *TFL1* (*VuTFL1*) homolog obtained from six genotypes (three determinate mutants, parent and two indeterminate varieties) were aligned using the program CLUSTAL X2 (Thompson et al. 1997) to identify base changes or SNPs. The sequence of the indeterminate parent was subjected to NCBI BLAST to identify homologous sequences from other genera and the coding region of most closely related genus was used to demarcate exons and introns. ExPASy Translate tool was used to predict the amino acid sequences of all the six genotypes. A phylogenetic tree of *TFL1*-related proteins was constructed using the NJ method with the program CLUSTAL X2 and the following sequences: *Lj_TFL* (AAQ93599); *Gm_TFL* (ACU00123); *PvTFLly* (ABR53775); *Ps_TFL* (AAR03725); *At_TFL* (NP_196004); *Bn_TFL* (BAA33415); *Ps_LF* (AAQ20811); *Am_CEN* (AAB36112.1); *Mt_TFL*(XP_003625808); *Ah_TFL* (AFP33421); *At_TS_FT/TFL1* (NP_193770); *At_FT* (BAA77838); *Os_CEN* (AAD42896); *At_Brother of FT/TFL-1* (NP_201010); *At_mother of FT/TFL-1* (NP_173250); *Cs_TFL* (NP_001275848); *Gm_DT1* (ADF30943), *Sl_SP* (NP_001233974) and *Vigna unguiculata* parent EC394763 (KJ569523). TreeView (Ver 1.6.6) was used to visualize the phylogenetic tree. ConSurf (Berezin et al. 2004) server tool was employed to estimate the evolutionary conservation of the mutant amino acid position in the predicted *VuTFL1* protein molecule based on the phylogenetic relations between homologous sequences.

Amino acid substitution effect prediction

The effect of coding non-synonymous mutation on protein function was predicted using different computational tools like SIFT (Kumar et al. 2009), PolyPhen-2 (Adzhubei et al. 2010), PANTHER (Thomas et al. 2006) and PredictSNP

Fig. 1 **a** Cowpea parent EC394763 with indeterminate growth habit exhibiting tendrillar growth and non-termination of main axis with terminal flowers. **b** Cowpea mutants "TCM418" (whole plant), "TCM420" and "TCM440" (showing close up of terminal shoot) with determinate growth habit exhibiting termination of main axis with floral buds

(Bendl et al. 2014). The effect of coding non-synonymous mutation on protein stability was also studied using computational algorithms like MUpro (Cheng et al. 2006), ProSMS, I. Mutant (Capriotti et al. 2005) and PoPMuSiC (Dehouck et al. 2009). MutPred (Li et al. 2009) tool was used to predict the molecular mechanism of disruption due to deleterious mutation.

Accession numbers

The sequence data obtained herein have been submitted to NCBI/GenBank data libraries with accession numbers KJ569520 (TCM418DT), KJ569521 (TCM440DT), KJ569522 (TCM420DT), KJ569523 (EC394763), KJ569524 (V-130) and KJ569525 (V-240).

Results

Identification of determinate mutant

The exotic indeterminate cowpea germplasm "EC394763" characterized with high yield, mid-early maturity, more pods per cluster and with field tolerance to major viral diseases (Fig. 1a) was used as the candidate line for improving plant type by mutation breeding. In M_2 generation, three determinate mutants ("TCM418", "TCM420" and "TCM440") (Fig. 1b) were identified in addition to other morphological mutants. These three mutants were characterized with terminal flowers, reduced plant height, reduced number of nodes, reduced number of leaves, and shortened internodal lengths (Table 1). No significant difference between the mutants and parent was observed with respect to days to flower though the mutants matured almost a week ahead of the parent. Due to reduction in the number of pod-bearing clusters consequent to the reduction in the number of nodes, the seed yield per plant in two of the mutants were low in comparison to the parent.

Identification of *Vigna unguiculata TFL1* (*VuTFL1*) homolog

The DNA from the three determinate mutants and parent was PCR amplified and a monomorphic band of about 890 bp was obtained using the primer pair TFL 1F/1R (Fig. 2). The sequence data of the monomorphic band on alignment showed four SNPs between the mutants and the parent. BLAST analysis indicated only one of the SNP to be present in the exonic region. BLAST alignment also showed maximum similarity (82 %) with

Table 1 Morphological characterization of determinate mutants and their parent in cowpea

Trait	EC394763 (parent)	Determinate mutants		
		TCM418	TCM420	TCM440
Plant height (cm)	106.6 ± 2.66	25.6 ± 1.03*	22.4 ± 0.87*	23.2 ± 0.80*
No. of nodes	16.4 ± 0.81	10.8 ± 0.73*	10.6 ± 0.24*	13.0 ± 0.55*
No. of branches	5.8 ± 0.58	4.8 ± 0.37*	4.6 ± 0.51*	4.6 ± 0.25*
Mean branch length (cm)	95.4 ± 5.35	23.0 ± 0.95*	20.2 ± 0.84*	20.4 ± 0.63*
Mean internodal length (cm)	6.15 ± 0.16	2.18 ± 0.05*	1.93 ± 0.04*	1.66 ± 0.06*
No. of leaves	50.6 ± 3.5	37.0 ± 1.87*	34.8 ± 1.98*	34.0 ± 2.17*
No. of pod clusters	24.6 ± 1.12	23.2 ± 1.85	21.6 ± 1.4*	17.6 ± 1.32*
Pod length (cm)	13.6 ± 0.25	14.6 ± 0.24	14.0 ± 0.32	14.4 ± 0.25
Seeds per pod	12.8 ± 0.37	13.4 ± 0.40	13.0 ± 0.32	13.2 ± 0.20
Seed yield per plant (g)	22.3 ± 1.83	20.1 ± 2.30	19.4 ± 1.02*	16.6 ± 2.3*
Days to flower	38.8 ± 0.43	38.6 ± 0.61	39.1 ± 0.22	39.2 ± 0.18
Days to maturity	71.1 ± 0.08	65.2 ± 0.09*	65.1 ± 0.04*	63.4 ± 0.12*

Values following ± indicate standard errors

* Significantly different from parent at 5 % probability

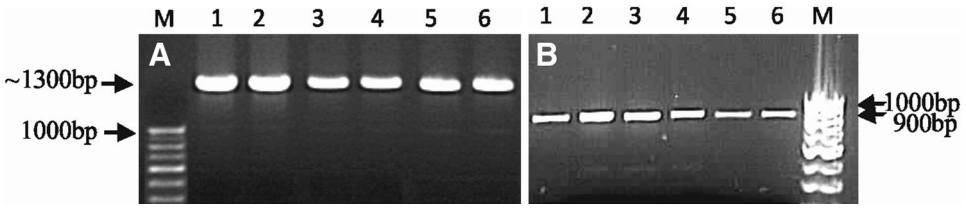

Fig. 2 **a** Amplification profile of complete *TFL1* homolog using primer pair TFL6F/TFL6R. **b** Partial *TFL1* homolog using primer pair TFL1F/TFL1R in cowpea. *Lane M*: 100 bp DNA ladder, *1*, "V-130"; *2*, "V-240"; *3*, "EC394763" (Parent); *4*, "TCM418"; *5*, "TCM420"; *6*, "TCM440"

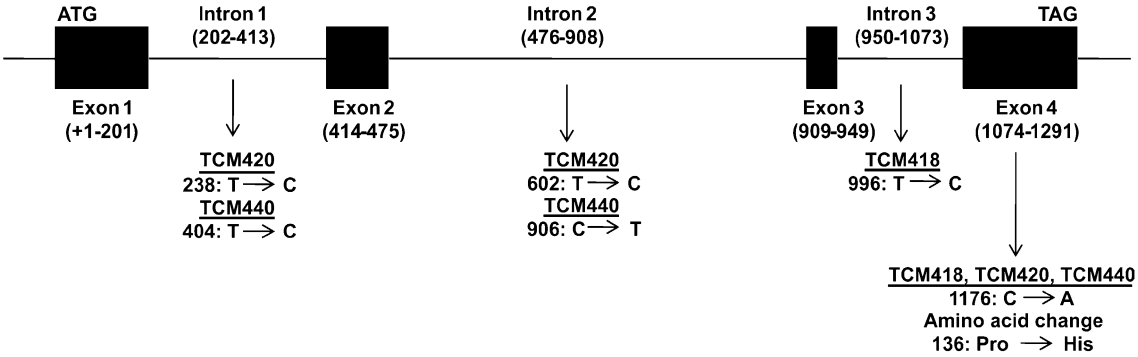

Fig. 3 Diagrammatic representation of cowpea *VuTFL1* homolog and the point mutations in the determinate mutants "TCM418", "TCM420" and "TCM440" following sequence analysis in comparison to the indeterminate parent "EC394763"

PvTFLly homolog. The end sequences of CDS region of *PvTFLly* gene was further used to design primers so as to amplify the entire *TFL1* homolog in cowpea. The primer pairs TFL 6F/6R successfully amplified a monomorphic band of about 1,300 bp in the three mutants, parent and as well in two indeterminate varieties ("V-130" and "V-240") (Fig. 2). Cloning and sequencing showed cowpea *TFL1* homolog, that is being reported for the first time, to be 1,291 bp long and could be referred to as *VuTFL1*. BLAST alignment of the *VuTFL1* resulted in identification of *P. vulgaris TFLly* gene to be the most closely related homolog with 90 % identity (97 % length) followed by *Glycine max Dt1* homolog with 82 % identity (79 % length). Alignment with *PvTFLly* coding region showed *VuTFL1* to be composed of four exons and three introns (Fig. 3). The nucleotide translation of 1,291 bp using

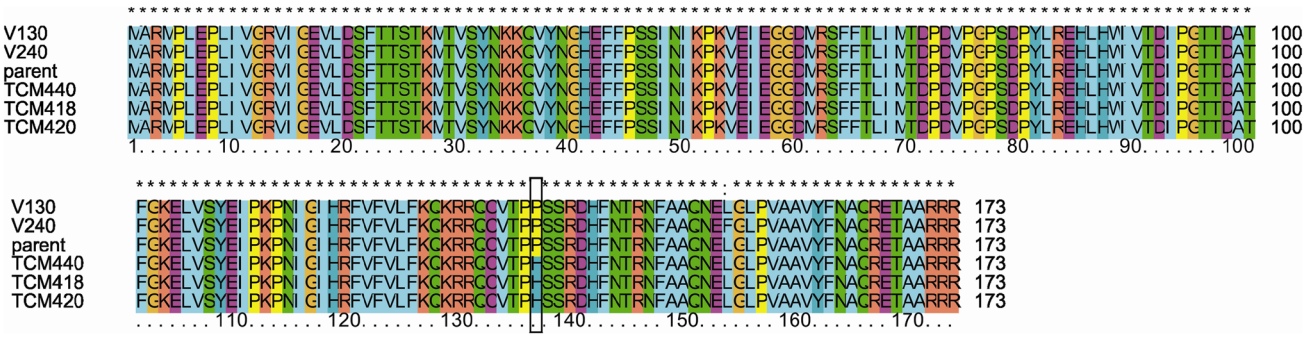

Fig. 4 Alignment of the deduced amino acid sequences of *VuTFL1* in determinate mutants ("TCM440", "TCM418", "TCM420"), indeterminate parent ("EC394763") and indeterminate varieties ("V-130", "V-240") generated with CLUSTAL X2 software. Identical amino acids are indicated by an *asterisk*. The amino acid change distinguishing the determinate from indeterminate growth habit is indicated by a *box*

Table 2 In silico prediction of mutated *VuTFL1* protein function and stability

S. no.	Software	Prediction	% Confidence/score	*P* value
1	SIFT	Affect protein function	79 %/0.00	Median sequence conservation: 3.06
2	PolyPhen-1	Deleterious	74 %	–
3	PolyPhen-2	Deleterious	68 %	–
4	Panther	Deleterious	79 %/SubPSEC = −4.3661	$P_{\text{substituted}} = 0.0146$
5	PredictSNP 1.0	Deleterious	87 %	–
6	MuPro	Decrease stability	−0.4508 (SVM)	–
		Decrease stability	−0.7653 (neural network)	
7	ProSMS	Destabilize	$\Delta\Delta G = -1.070$	$P_{\text{destabilizing}} = 0.55$
8	i.Mutant	Decrease stability	8 (reliability index)	–
9	PoPMuSiC v2.1	Destabilizing	$\Delta\Delta G = 0.94$ kcal/mol	–
10	MutPred	Deleterious	Loss of glycosylation ($P = 0.0147$)	$P_{\text{deleterious}} = 0.515$

ExPASy tool predicted *VuTFL1* protein to be 173 amino acids long (Fig. 4).

Identification of SNP distinguishing the determinate mutants

The alignment of sequences of the three determinate mutants and parent showed a total of six SNPs; two each in intron 1 and intron 2, and one each in intron 3 and exon 4, respectively (Fig. 3). Inclusion of sequences of two other indeterminate genotypes in alignment showed a total of 12 SNPs; two in intron 1, five in intron 2, one in intron 3 and four in exon 4. Among the four exonic SNPs, one is non-synonymous while two are synonymous and one is conservative substitutions (Fig. 4; Online Resource 2). The non-synonymous substitution in exon 4 at position 1,176 was the only SNP that distinguished the determinate mutants from the indeterminate parent and varieties. The cytosine base (C) in the indeterminate plant types was substituted with adenine (A) base in mutants resulting in predicted change of single amino acid at position 136 from proline (Pro) in wild types to histidine (His) in mutants.

Prediction of mutation on protein function/stability

The effect of single amino acid substitution on protein function/stability was predicted to be deleterious and unstable using a variety of computational tools as shown in Table 2, leading to loss-of-function hypothesis. The molecular mechanism of functional disruption arising out of the mutation was predicted by MutPred and was mainly attributed to loss of glycosylation at P136 ($P = 0.0147$). The neural network-based algorithm of ConSurf predicted that the 'Pro' amino acid at position 136 is an exposed, highly conserved and functional residue on the protein surface (Online Resource 3).

Phylogenetic analysis

The phylogenetic tree of *TFL1*-related proteins constructed using NJ method with the program CLUSTAL X2 showed

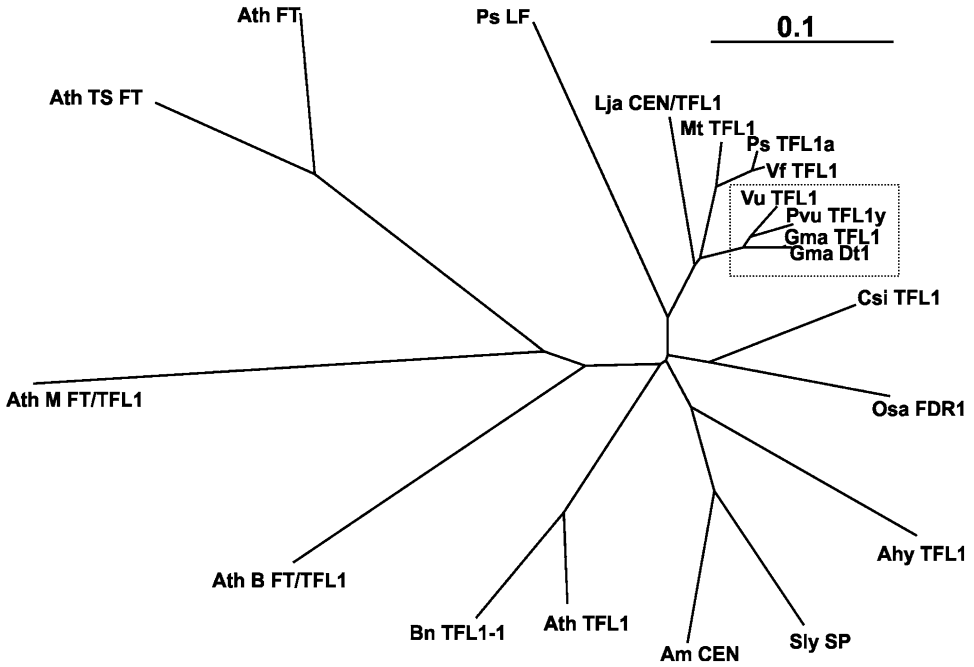

Fig. 5 Phylogenetic tree of *TFL1*-related proteins constructed using NJ method with the program CLUSTAL X2 in radial tree view. The NCBI accession number of sequences used are indicated in parenthesis: *Lotus japonicus-Lja_TFL1* (AAQ93599); *Glycine max-Gma_TFL* (ACU00123); *Phaseolus vulgaris-PvuTFLly* (ABR53775); *Pisum sativum- Ps_TFL1a* (AAR03725); *Arabidopsis thaliana-Ath_TFL1* (NP_196004); *Brassica napus-Bn_TFL1-1* (BAA33415); *Pisum sativum-Ps_LF* (AAQ20811); *Antirrhinum majus-Am_CEN* (AAB36112.1); *Medicago truncatula-Mt_TFL1*(XP_003625808); *Arachis hypogaea-Ahy_TFL1*(AFP33421); *Arabidopsis thaliana-Ath_TS_FT/TFL1* (NP_193770); *Arabidopsis thaliana-Ath_FT* (BAA77838); *Oryza sativa-Osa_FDR1* (AAD42896); *Arabidopsis thaliana-Ath_Brother of FT/TFL-1* (NP_201010); *Arabidopsis thaliana-Ath_mother of FT/TFL-1* (NP_173250); *Citrus sinensis-Csi_TFL1* (NP_001275848); *Glycine max-Gma_DT1* (ADF30943), *Solanum lycopersicum-Sly_SP* (NP_001233974) and *Vigna unguiculata-VuTFL1* (KJ569523)

that *VuTFL1* had very high sequence homology with *PvTFLly* followed by *Glycine max TFL* and *Dt1*, *Pisum sativum TFL1a*, *Vicia faba TFL1*, *Medicago truncatula TFL1* and *Lotus japonicus TFL* (Fig. 5). The amino acid sequence alignment of *TFL1*-related proteins showed that the amino acid position at site of mutation (136) was highly conserved across the genera (Online Resource 4).

Discussion

Isolation and characterization of genes associated with stem growth habit are very important for cowpea germplasm assessment and breeding, as stem termination has great effects on plant height, flowering period, node production, maturity, water-use efficiency and yield. The difficulty in distinguishing determinate from indeterminate plant types under short photoperiod conditions or under adverse growing conditions (Bernard 1972), could be overcome by developing molecular markers associated with the trait. Hence, understanding the molecular mechanisms governing growth habit becomes imperative in restructuring cowpea ideotype for development of suitable varieties.

Mutants serve as means for identifying genes that control developmental decisions in plants like flowering (Coen 1991) and crops with improved traits are being developed by screening for mutations induced in candidate genes (Julio et al. 2008; McCallum et al. 2008). The rationale being that when a gene controlling a specific developmental process is mutated, the process controlled by the mutated gene is likely to be specifically disrupted (Alvarez et al. 1992). *TFL1* plays a significant role in determination of floral meristem identity in *A. thaliana* and has been found to be highly conserved among various genera (Tahery et al. 2011). A deficient mutant in the function of this gene results in formation of terminal flower leading to determinate growth habit (So Yeon et al. 2004).

Highly penetrating radiations such as X-rays and gamma rays have low LET and mediate their effects through an indirect mechanism that involves splitting of water molecules causing free radical formation. In addition to deletion, indirect damage to DNA by ionizing radiations like gamma rays may cause point mutations in low frequencies resulting in single base substitutions (Sato et al. 2006). The utility of gamma rays in inducing determinate growth habit in cowpea has been previously demonstrated (Pandey

and Dhanasekar 2003). The identification of determinate mutants in the present study reiterates the utility of gamma rays in inducing useful mutants. The single nucleotide mutation leading to determinacy could have resulted as a spinoff of indirect damage to DNA by the gamma rays. The determinate mutants were characterized with reduced plant height due to reduction in the number of nodes as well as shortened internodal lengths, less number of leaves, branches and flowers which corroborate with previous reports (Alvarez et al. 1992; Schultz and Haughn 1993). Even though the three mutants were phenotypically similar, differences were observed in some morphological parameters (Table 1) other than terminal flowering habit. This could be explained in terms of the randomness of mutation, wherein some genes in addition to the *TFL1* gene could have mutated resulting in the difference. Moreover, all the three mutants carried the same exonic SNP though there were different mutations in the intronic regions suggesting that the point of mutation could be a highly mutable region within the gene. Foucher et al. (2003) also described similar situation in peas wherein two determinate mutants carried 3 point mutations at the same position in addition to two other SNPs in *PsTFL1a* gene. The mutation in the present investigation, however, had no effect on days to flowering. In peas also it was demonstrated that mutations in *PsTFL1a* cause determination of main apex without affecting flowering time, whereas mutations in *PsTFL1c* caused early flowering without affecting determination (Foucher et al. 2003). Determinate growth habit has been reported to be a recessive trait in various crops (Koinange et al. 1996). In the present study also, the preliminary data suggests determinacy to be recessive in nature. The number of determinate plants within the M_2 progeny and their true breeding behaviour in M_3 suggested the recessive nature of mutation in these three determinate mutants (Dhanasekar and Reddy, Unpubl Res).

The effect of genetic mutation on phenotype is of significant interest in genetics. A non-synonymous SNP could potentially affect the function of the protein, subsequently altering the carrier's phenotype. So once a mutation is identified, it is worthwhile to estimate the mutation's likelihood of damaging the protein (Flanagan et al. 2010). Functional and translational genomics in cowpea being limited by its recalcitrance to genetic transformation, the computational and bioinformatics tools offer means for making in silico predictions and inferences about protein structure and function. The degree to which an amino (or nucleic) acid position is evolutionarily conserved is strongly dependent on its structural and functional importance; rapidly evolving positions are variable while slowly evolving positions are conserved. Thus, conservation analyses of positions among members from the same family often reveal importance of each position for the protein (or nucleic acid)'s structure or function (Thomas et al. 2003). Eighty-eight percent of the mutations have also been found to affect highly conserved residues (Flanagan et al. 2010).

Software tools that use protein homology information to predict damaging lesions are available for this purpose (Thomas et al. 2006; Kumar et al. 2009; Adzhubei et al. 2010; Bendl et al. 2014). Sequence homology-based approach to predict damage to a protein caused by random missense mutation can be applied generally across organisms (Henikoff and Comai 2003). Flanagan et al. (2010) reported that the sensitivity of tools such as SIFT (Sorting Intolerant From Tolerant) and PolyPhen (Polymorphism Phenotyping) have been found to be reasonably high (69 and 68 %, respectively). Both programs were significantly better at predicting loss-of-function mutations than gain-of-function mutations (SIFT, $P = 0.001$; PolyPhen, $P \leq 0.0001$). PredictSNP tool, a consensus classifier integrates six best performing tools, resulting in a significantly improved prediction performance (Bendl et al. 2014). Single amino acid mutations can also significantly affect the stability of a protein structure. The methods for predicting protein stability changes resulting from single amino acid mutations generally rely on energy functions (Capriotti et al. 2004), while the machine learning approach captures complex local and nonlocal interactions that improve the accuracy of prediction affecting protein stability (Cheng et al. 2006). In the present study, all the computational tools predicted the mutation to be damaging as shown in Table 2. In the neural network-based algorithm of ConSurf tool, the evolutionary rate is estimated based on the evolutionary relatedness between the protein (DNA/RNA) and its homologs. In the present investigation, ConSurf predicted the 'P' amino acid at position 136 to be an exposed, highly conserved and functional residue on the protein surface whose non-synonymous substitution could have highly probable implications in the protein function. The protein stability prediction tools also predicted the SNP mutation as destabilizing leading to loss of protein function hypothesis. The molecular mechanism of functional disruption arising out of the mutation was mainly attributed to loss of glycosylation at P136 ($P = 0.0147$) along with others (Online Resource 5) as predicted by MutPred.

The *TFL1* gene is known to maintain the indeterminate growth habit by inhibiting the differentiation of vegetative apical meristems to floral meristems. Any mutation in the *TFL1* gene with a functional loss is expected to remove the repression resulting in termination of vegetative apical growth with floral buds. In the present study, it is evident that the indeterminate parent "EC394763" was mutated in the *TFL1* gene loci and the SNP identified in exon 4 with loss of function prediction could have resulted in these determinate mutants "TCM418", "TCM420" and "TCM440". Foucher et al. (2003) also reported

similar results in *P. sativum* while studying three homologs of *TFL1/CEN* family (*PsTFLIa, b* and *c*). Sequencing of *PsTFL1a* in three determinate lines revealed mutations in introns and exons in comparison to the wild type, some of which were silent mutations, but others significantly modified the *PsTFL1a* structure leading to a non-functional protein responsible for determinate growth habit in *det* mutants.

Amino acid changes have implications in enzyme function and thereby on the phenotype. Foucher et al. (2003) reported that single amino acid changes resulted in determinate growth habit phenotype in two different pea mutants. Similar findings have also been reported in *Arabidopsis* (Oshima et al. 1997) and tomato (Pnueli et al. 1998). Even between homologous genes, a single amino acid substitution promotes change in enzymatic function as accounted by Hanzawa et al. (2005) who studied two 60 % identical *Arabidopsis* CEN/TFL1-like genes homologs involved in clear and opposite functions and showed that swapping a single amino acid was sufficient to convert a repressor to an activator of flowering and vice versa. To our knowledge, the mutation leading to amino acid substitution of Pro-136 with His in cowpea described in this study has not been previously reported in any other species.

Ahn et al. (2006) reported that fourth exon of *TFL1* gene has critical role in determining determinacy. They studied the structural differences of segment B (residues 128–145) encoded by the fourth exons of *FT* and *TFL1* in *Arabidopsis* that was critical for *FT* versus *TFL1* activity in vivo and found that these residues formed an external loop that was in close proximity to, and ultimately led directly to the residues that form part of potential ligand-binding site. In the present study, also the mutation is at position 136 (*VuTFL1*) that corresponds to position 139 of *TFL1* and is expected to be integral part of the external loop affecting the potential ligand-binding site. Moreover, substitution of neutral 'Pro' with positively charged 'His' could potentially alter the surface properties of the protein influencing ligand binding. Superimposition of the predicted structure using ExPasy on *TFL1* protein structure shows the mutation to be in the external loop potentially affecting protein interaction. The molecular mechanism of mode of action of *TFL1* even though is obscure; the recent findings suggest that *TFL1* is involved in transcriptional repression mechanisms that regulate floral meristems identity genes that are also *FT* targets. *TFL1* and *FT* interact with FD and also interact with an unknown corepressor and a coactivator, respectively (Hanano and Goto 2011). The mutation in the present study in *VuTFL1* gene possibly prevents its interaction with the hypothetical corepressor resulting in removal of transcriptional repression and activation of *FT* leading to terminal flowering.

The *VuTFL1* sequence corresponding to the parent cowpea line "EC394763" was aligned with *TFL1/CEN*-related sequences obtained from the databases. The phylogenic tree based on amino acid similarity revealed *VuTFL1* to be '*TFL1*' and not '*CEN*' like. *VuTFL1* formed a clade with *PvTFLly* and *G. max TFL1* and *Dt1* sequences. The BLAST analysis of 173 amino acid-long *VuTFL1* homolog exhibited 95 and 94 % homology with *PvTFLly* and *G. max* (*TFL1* and *Dt1*) proteins, respectively, of similar lengths. Based on clustering pattern and length of the proteins, it could be inferred that these *TFL1* proteins and the 174 amino acid-long *TFL1* proteins of *Vicia faba*, *Medicago truncatula*, *P. sativum* and *Lotus japonicus* with whom they exhibited identities ranging from 88 to 90 %, might have common evolutionary origin. The 177 amino acid-long *A. thaliana TFL1* protein was distantly related and exhibited only 74 % similarity with *VuTFL1* protein. Foucher et al. (2003) also reported an identity of 72 % between *PsTFL1a* and *Arabidopsis TFL1*. Alvarez et al. (1992) have suggested that TFL gene may be one of a class responsible for evolutionary changes between indeterminate and determinate growth.

Thus, it is concluded that the functionally significant novel substitution mutation obtained in *VuTFL1* gene in the present investigation could be hypothesized to affect determinacy in the cowpea mutants. Development of suitable regeneration protocols in this hitherto recalcitrant crop and conducting subsequent complementation assay in mutants or overexpressing assay in parents could decisively conclude the role of the SNP in regulating determinacy in these cowpea mutants. Furthermore, the nucleotide position of mutation could be exploited by site-directed mutagenesis for developing determinate plant types in elite genotypes of cowpea.

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