PTC-1: A homologue of *TFL1/CEN* involved in the control of shoot architecture in *Beta palonga*

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A terminal flower mutant phenotype of *Beta palonga*, a leafy vegetable, isolated earlier has been characterized. Micromorphological and histological study of the normal and mutant phenotype reveals physiognomic similarity of the mutant phenotype with terminal flower mutant of *Arabidopsis*. To investigate the regulation of meristem identity and control of flower development of the mutant phenotype, degenerated primers were designed from the highly conserved region of floral identity gene *TFL1/CEN*. This resulted in a 238 base pair-specific amplified cDNA product by RT–PCR, named as PTC-1. Sequence analysis followed by BLAST showed high homology of the PTC with *TFL1/CEN*-like gene, indicating the presence of *TFL1/CEN* homologue in *B. palonga*. Southern analysis indicates alteration of the genomic sequence of the mutant of *B. palonga*. The significance of the terminal flower is discussed.

Keywords: *Beta palonga*, inflorescence, shoot architecture, terminal flower.

Beta palonga (Palak in Hindi; Palong in Bengali)[1], consumed as a leafy vegetable, is cultivated during winter under tropical climatic condition throughout India. In an earlier attempt[2], seeds of *B. palonga* were irradiated with ionizing radiation to induce variability. The terminal flower mutant[3] is a product of that effort. Apart from the terminal flower, the mutant has several characteristic features like early bolting, thick leaves, and rigid and less compact inflorescence compared to the normal plant[3].

There has been a marked advance in the understanding of floral initiation and inflorescence development from studies of mutant plants displaying altered inflorescence development. Terminal flowers are found in the *Arabidopsis* terminal flower1 (*TFL1*) mutant[4–8] and in the *Antirrhinum centroradialis* (*CEN*) mutants[9]. In *TFL1* and *CEN* mutants, central flowers are not produced initially, indicating that the inflorescence meristem (IM) is active during early reproductive development. However, after developing a few flowers, the IM is converted to floral meristem (FM) and ultimately forms a terminal flower at the apex. Results of genetic analysis have indicated that *TFL1* or *CEN* may be necessary to maintain indeterminate inflorescence by interacting with other floral-meristem identity genes, *LFY/FLO* and *AP1/SQUA*. The *TFL1* and *CEN* mutants differ in some respects; for example, *CEN* inflorescence has a single terminal flower, whereas *TFL1* apices usually have two or three flowers. In addition, *TFL1* mutant flowers early while *CEN* mutant does not[8,10,11].

TFL1 and *CEN* show a similarity in deduced amino acid sequence, expression pattern and mutant phenotype[4,6–9]. *CEN* has been shown to act like *TFL1* by indirectly interacting with *FLO*[12], an *Antirrhinum* counterpart of *LFY*. The TFL1 and CEN proteins belong to a family of proteins in mammals that were initially characterized as phosphatidyl-ethanolamine-binding proteins (PEBP)[13–16]. A human PEBP (RKIP: Raf Kinase Inhibitor Protein) has been shown to specifically regulate the Raf-1/MEK/ERK signalling pathway[17], suggesting a possible role for *TFL1* in disruption of a floral-promotive signalling pathway in *Arabidopsis*. Over-expression of *TFL1* causes a lengthening of the vegetative phase, increased secondary inflorescence production and delay in flowering[4,6,11,18].

Incidentally, the terminal flower mutant of both *Arabidopsis* and the material under study, *B. palonga*, appeared to be physiognomically similar. The mutant of *B. palonga*, therefore, provides a unique opportunity to investigate this system, taking clues from *Arabidopsis*.

In an earlier attempt it was established that the inheritance pattern of this terminal flower character in reciprocal cross between mutant and normal[3] followed by F_2 and testcross generations; segregating ratio was 3 : 1 and 1 : 1 respectively. In the present investigation the mutant was characterized at phenotypic level and the mutant gene identified through molecular techniques and partially sequence (PTC-1; DQ849290).

Materials and methods

Plant materials

Beta palonga, a member of the family Chenopodiaceae and an X-ray induced terminal flower mutant[3] formed the materials of the present investigation. The seed stock of

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B. palonga and the mutant was maintained in the experimental farm of Bose Institute at Madhyamgram, 24-Parganas (North), West Bengal, India.

Measurement of day length and temperature

The day length and temperature data of the last five years (2000–05) were collected from 'Regional Meteorological Centre', Dum Dum Airport Division, located close to the experimental farm of Bose Institute.

Histology

Histological study was done by following the routine histological procedure[2] and a rotary microtome was used for sectioning.

Scanning electron microscopy

For scanning electron microscopy (SEM), inflorescences were cut into suitable size, fixed separately in 3% glutaraldehyde in phosphate buffer (0.1 M and pH 6.8) for 2 h. Materials were washed with the same phosphate buffer and post-fixed with osmium tetra oxide (1% v/v) followed by drying with ethanol grade (70 to 100% alcohol). Dehydrated materials were then transferred to amyl acetate before critical-point drying with liquid CO_2. The inflorescences were mounted on aluminum stubs and coated with gold. The specimens were observed in Phillips, Holland, SEM 500 scanning electron microscope. Electron micrographs were taken at 45° angle with a Robort-star camera.

RT–PCR analysis

Total RNA was extracted from approximately 2 mg inflorescence tissue using the RNeasy mini kit (QIAGEN, USA). Total RNA was treated with RNAase-free DNAase, followed by a heat inactivation step (75°C for 5 min). Reverse transcription for each sample was carried out in a 20 μl reaction mixture using a first strand cDNA synthesis kit (Roche, USA). Second strand synthesis was carried out using degenerated oligonucleotide primers (Table 1) designed specifically from conserved part of the *TFL1* and its homologue. PCR was performed using 100 ng of cDNA and 10 pM of reverse and forward primers, 2.5 units of *Taq* polymerase and 100 μM of each dNTP in 25 μl of reaction mixture. Amplification was carried out in a Thermal Cycler (PTC-100, M J Research, Inc, USA) with denaturation at 94°C for 30 s, annealing at 50°C for 30 s and polymerization at 72°C for 1 min, for the first ten cycles followed by denaturation at 94°C for 30 s, annealing at 58°C for 30 s and polymerization at 72°C for 1 min for 20 cycles to improve the binding of the primer to the template.

DNA sequencing

DNA sequencing was done using M-13 forward (F) and reverse (R) primer at DNA Sequencing Facility, UDSC, Department of Biochemistry, University of Delhi South Campus, New Delhi, India. Six different clones were sequenced separately to avoid the technical/PCR mutational error.

Southern blot analysis

DNA was extracted from young leaves of both control and mutant plant using a modified CTAB method[19]. Five microgram samples of DNA from control and mutant plant were digested with three different restriction enzymes *Eco*RI, *Hin*dIII and *Eco*RV, electrophoresed in 0.8% agarose gels and transferred to nylon membrane (Roche) for Southern analysis. The PTC-1, a 238 bp fragment were labelled with [α^{32}P] dCTP (BRIT, Mumbai, India) using Random Priming DNA labelling kit (New England Biolabs).

Southern analysis of amplified PCR products of control and mutant *B. palonga* was performed with PTC-F and PTC-R primers and 1.2% agarose gel, and PTC-1 fragment was used as a probe; other conditions were the same.

Table 1. Primers used for PCR in this study

Primer	Sequence (5′ to 3′)[a,b]
F1	5′ GACT**GGATCC**AA(C/T)GGICA(C/T)GA(A/G)(C/T)TITT(C/T)CC 3′
F2	5′ GACT**GGATCC**(A/C)GIGA(A/G)CA(C/T)(C/T)TICA(C/T)TGG 3′
R1	5′ CTGC**AAGCTT**C(G/T)IGCIGC(A/G)GT(C/T)TCIC(G/T)(C/T)TG 3′
R2	5′ CTGC**AAGCTT**AA(A/G)AAIACIGCIGCIAC(A/T)GG 3′
AP	5′ CTGAGGATCCGTCGACTAGTAC 3′
PTC-F	5′ TAGT**GAGCTC**ATTCCAGGCACAACAGATGCC 3′
PTC-R	5′ GATT**AAGCTT**CGCGGCGACTGGAAGTCCTA 3′

[a]I, Ionosine.
[b]Bold underline sequence part represents restriction site.

PCR analysis

PCR was performed using 100 ng of DNA and 10 pM of forward and reverse primers (PTC-F and PTC-R; Table 1), 2.5 units of *Taq* polymerase and 100 µM of each dNTP in 100 µl of reaction mixture. Amplification was carried out in a Thermal Cycler (PTC-100, M J Research, Inc.) with denaturation at 94°C for 30 s, annealing at 62°C for 30 s and polymerization at 72°C for 1 min, for 30 cycles.

Bioinformatics study

Bioinformatics study was done mainly using the PubMed (http://www.ncbi.nlm.nih.gov/PubMed/) service and some other server and software programs, viz. BioEdit version 5.0.9, Department of Microbiology, North Carolina State University, USA; Restriction Mapper (http://www.restrictionmapper.org/); and CLUSTAL W (http://www.ebi.ac.uk/clustalw/).

Statistical analysis

Statistical analysis was done using the computational software, Microcal Origine version 6.0, Microcal software, Inc. and also the statistical software SPSS 10.0.5, SPSS Inc.

Results

Description of the terminal flower phenotype

In the mutant, the terminal flowers were not produced initially, but after developing a few flowers, the IM was converted to FM and ultimately formed a terminal flower at the apex. Normal plant had compact spike inflorescence where IM had an indeterminate growth. The mutant rachis was more rigid compared to control and the inflorescences were less compact having lesser number of flowers with determinate growth. In the mutant, flowers were bigger compared to control (Figure 1 *a* and *b*). Histologically and micro-morphologically (SEM study) it has been observed that in the mutant, the apical meristem clearly manifested the conversion of indeterminate to determinate growth, which is a characteristic feature of terminal flower phenotype (Figure 1 *c–f*).

Life cycle

The life cycle of *B. palonga* has four distinct phases: juvenile phase (JV), adult vegetative (AV), early reproductive phase (ER) and late reproductive phase (LR). During transition to flowering (ER), a primary inflorescence is produced (bolting). Late inflorescence (LR) primordia exclusively give rise to flowers. During flowering, apical dominance decreases allowing the secondary inflorescences (from the meristems in the axils of the rosette leaves) to develop. After maturation of the seeds, depending upon the temperature, mature plants gradually dry up.

B. palonga is a long day plant and completes its life cycle within 120 to 140 days. The mutant of *Beta* flowers early and completes its life cycle in a comparatively short time-span (90 to 110 days). Data on day length and temperature during the last five years (2000–05) show that there is a relationship among day length, temperature and bolting of the normal plant (Figure 2). In *B. palonga*, bolting occurs during the end of December to the first week of January under this agroclimatic condition (between 70 and 80 days), when the day length and temperature gradually increased day by day. In the case of the mutant, it bolts early (between 50 and 55 days). A stagger planta-

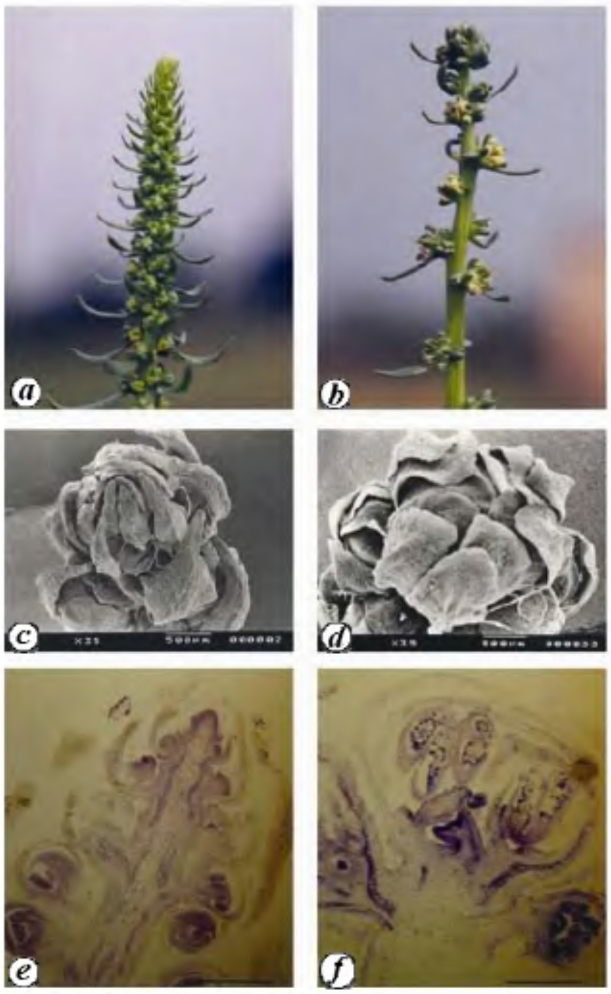

Figure 1. Normal and terminal flower mutant of *Beta palonga*. ***a***, Compact spike of normal *B. palonga*; ***b***, Less compact spike of mutant *B. palonga* with terminal flower; ***c***, SEM of normal inflorescence. ***d***, SEM of mutant showing terminal flower. ***e***, Histology (LS) of normal inflorescence showing apical meristem. ***f***, Histology (LS) of mutant inflorescence showing presence of terminal flower.

tion experiment (three different plots were sown at ten days interval) of the mutant showed that it bolts between 50 and 55 days irrespective of the date of sowing, day length and temperature.

Designing of the degenerated primers

To obtain genomic information and expression profile of *TFL1/CEN*-like gene from the normal and mutant *B. palonga*, a multiple alignment was performed using CLUSTAL W (1.82) multiple sequence alignment program with translated amino acid sequence of the *TFL1* (BAB08610), *CEN* (CAC21563), *CET2* (AFI45261), *CET4* (AFI45262) and *SP* (AAC26161) genes from the NCBI databank. Four degenerated primers (F1, F2, R1 and R2) were designed from the highly conserved sequence region of these genes (Table 1).

Amplification of TFL1 homologue

The first strand cDNA was synthesized from the total RNA isolated from inflorescence meristem of normal *B. palonga* using an oligo-dT anchored primer. Then, using the overhang sequence of the oligo-dT anchored primer (AP, Table 1) and F1, the first cycle of amplification was done. The second cycle of amplification (nested-PCR) was done using F2 and R2 primers. The amplified product of the first round of PCR cycle was used as a template here. In the present experiment the R1 primer does not work properly. After second cycle, a ~250 base pair specific amplified DNA fragment was obtained, which fitted with the expected size of *Arabidopsis TFL1* cDNA clone with the same set of primers (F2 and R2).

The ~250 base pair specific amplified cDNA product was cloned into the pBluescript SK+ vector for further sequence analysis. Six different clones (A-121 to A-126) were analysed through automated DNA sequencing.

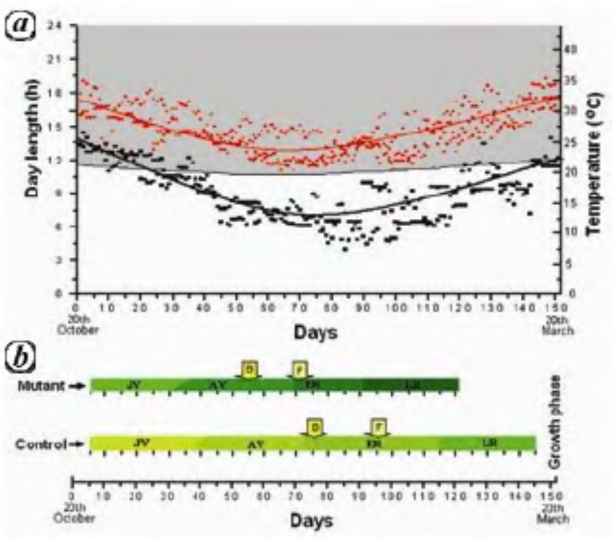

Figure 2. ***a***, Bolting and flowering pattern of normal and mutant *B. palonga* in the background of day length and temperature. Grey colour indicates dark phase and white indicates illuminated phase. Red spots indicate maximum temperatures and black spots indicate minimum temperatures. ***b***, Solid bar represent the life cycle pattern of control and mutant *B. palonga*. JV, Juvenile phase; AV, Adult vegetative phase; ER, Early reproductive phase; LR, Late reproductive phase; B, Bolting and F, Flowering.

Translate the partial sequence of PTC (Palong TFL1 or CEN-like gene)

The sequence information obtained from six different clones was alike. The nucleotide sequence was translated into six frames using BioEdit version 5.0.9. Translated amino acid sequence of all the possible open reading frames, among these frames the +1 frame is the right forms since this has highest amount of homology with the *Arabidopsis TFL1* gene. Additionally, absence of stop codon in its entire length proves that it may be part of a functional gene. The partial amplified *TFL1* homologue of *B. palonga*, a sequence with 79 amino acids, was renamed as *PTC-1* (*PTC*: Palong *TFL1/CEN*-like gene; NCBI accession no. DQ849290).

Multiple alignment with homologous sequences

To predict homology from the partial sequence of the *PTC*, a BLASTX 2.2.10 program was run under the NCBI server using the sequence information of *PTC-1*. The results showed that the *PTC* had above 65% identity at the amino acid level with *TFL1/CEN*-like genes isolated from different plants (Table 2). The highest level of identity was found with *TFL1/CEN* homo or ortholog proteins from *Pisum sativum* (AAR03725.1; 76% identity). To determine the evolutionary relationships between PTC and other TFL1/CEN-like proteins, a phylogenetic tree was constructed using the predicted protein sequences with the help of CLUSTAL W (1.82) software, which was also used for multiple alignment (Figure 3). The phylogram was constructed with CLUSTAL W (1.82) using phylip phylogram constructing mode. The generated phylogram is represented in Figure 4.

Southern blot analysis of normal and mutant using PTC-1 as a probe

In the Southern hybridization experiment, the genomic DNA was digested using three different hexacutters. *Eco*RI, *Hin*dIII and *Eco*RV were the restriction enzymes chosen on the basis of the virtual digestion result of *PTC-1* linear sequence. Southern hybridization was performed

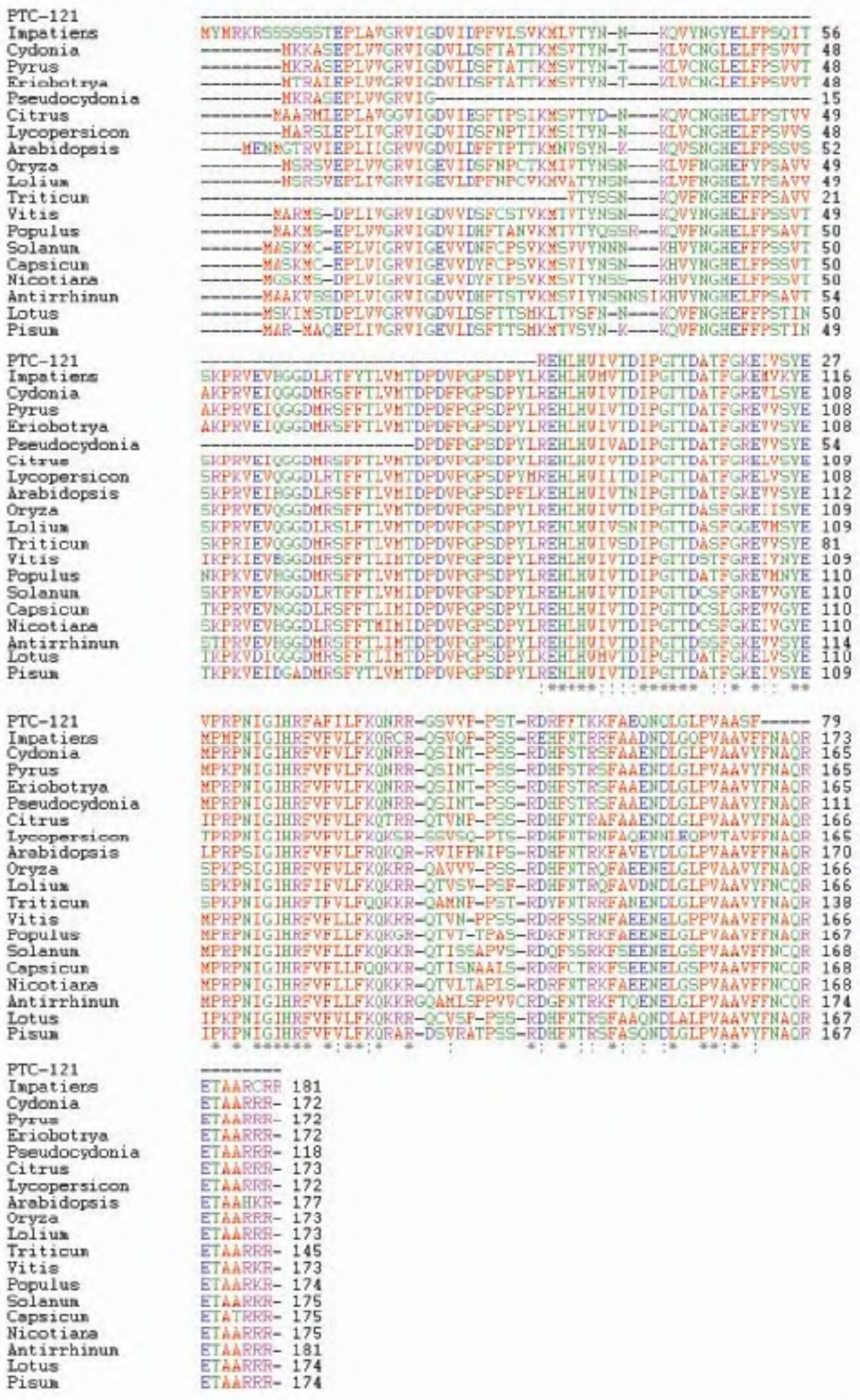

Figure 3. CLUSTAL W (1.82) multiple sequence alignment of the partial sequence of *TFL1* and *CEN*-like gene of normal *B. palonga* (*PTC-121*) and some related homologues from different plant species.

using *PTC-1* as a probe. As shown in Figure 5, two bands were detected in the case of normal where DNA was digested with *Eco*RI and *Eco*RV, but with the same restriction enzymes the mutant showed a single band. On the other hand, *Hin*dIII digested DNA of normal and mutant showed a single ~1.9 kb band in both cases.

Amplification of control and mutant genomic DNA

Control and mutant genomic DNA were amplified with PTC-F and PTC-R (Table 1). Two bands (~250 bp and 1.26 kb) appeared in case of control, but the mutant showed only one band (~250 bp) after gel electrophoresis of the amplified products (Figure 6). To reconfirm the amplified product, Southern hybridization was performed using *PTC-1* as a probe. A similar result was obtained as in the case of PCR amplification (Figure 7). The sequence results of the amplified bands showed two homologous sequences in which the 1.26 kb band of control *B. palonga* had a 953 kb intron (data not shown).

Discussion

Determinacy/indeterminacy of the inflorescence is usually a stable character and is often used in species identification. In several species, occasional conversions of an indeterminate into a determinate one have been reported, but the heritability of such transformations has rarely been studied. Only in a few species have these transformations been shown to be due to mutations[4,8,9,20]. In this context, the material under study, a leafy vegetable, appears to be a new candidate.

Table 2. Identity between translated *PTC-121* sequence and *TFL-I/CEN*-like genes isolated from different plants

Plant	NCBI accession no.	Identity (%)	*E*-value
Antirrhinum majus	AAB36112.1	69	$2e^{-27}$
Arabidopsis thaliana	AAM27952.1	68	$6e^{-25}$
Capsicum annuum	AAZ66798.1	65	$5e^{-25}$
Citrus sinensis	AAR04684.1	75	$3e^{-30}$
Cydonia oblonga	BAD10964.1	74	$5e^{-30}$
Eriobotrya japonica	BAD10966.1	75	$2e^{-30}$
Impatiens balsamina	CAI61982.1	70	$3e^{-27}$
Lolium perenne	AAG31808.1	68	$1e^{-25}$
Lotus corniculatus	AAQ93599.1	73	$3e^{-29}$
Lycopersicon esculentum	AAO31795.1	69	$2e^{-27}$
Nicotiana tabacum	AAD43531.1	74	$1e^{-28}$
Oryza sativa	ABA95827.1	73	$4e^{-29}$
Pisum sativum	AAR03725.1	76	$7e^{-29}$
Populus nigra	BAD22600.1	73	$9e^{-30}$
Pseudocydonia sinensis	BAD10965.1	73	$9e^{-30}$
Pyrus communis	BAD10963.1	74	$5e^{-30}$
Solanum tuberosum	ABC24691.1	66	$6e^{-26}$
Triticum aestivum	CAE53887.1	69	$2e^{-29}$
Vitis vinifera	AAM46142.1	75	$3e^{-30}$

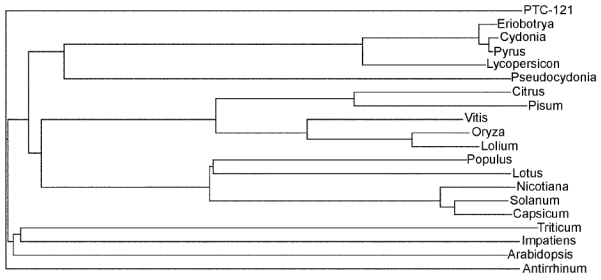

Figure 4. Phylogram tree generated from sequence information of *PTC-1* and its homologue.

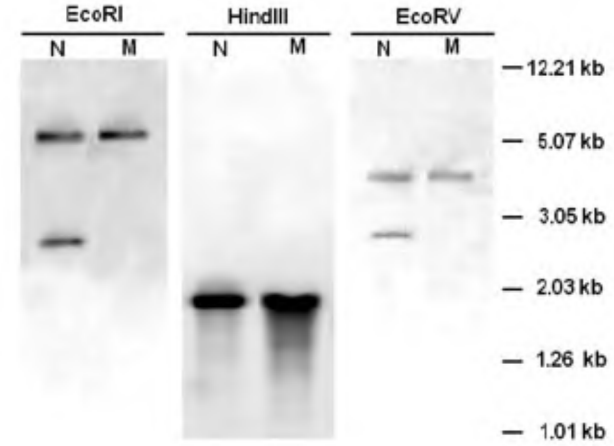

Figure 5. Southern blot analysis of normal and mutant *B. palonga* *Eco*RI, *Hin*dIII and *Eco*RV digested genomic DNA using PTC-1 fragment as a probe. N, Normal; M, Mutant.

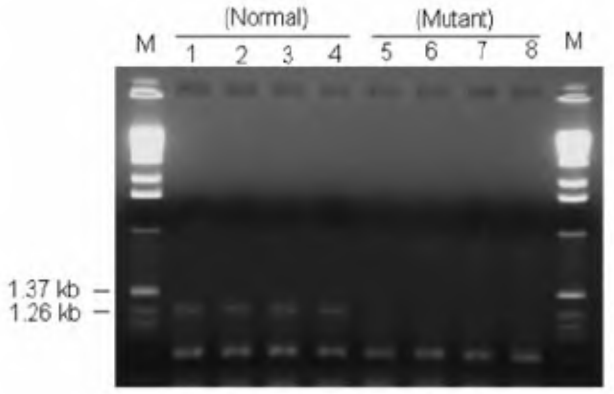

Figure 6. PCR amplification of control and mutant genomic DNA of *B. palonga* with PTC-F and PTC-R primers. M, Molecular marker (lambda DNA/BstE II digest).

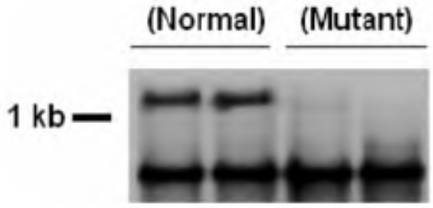

Figure 7. Southern blot analysis of PCR-amplified products from control and mutant *B. palonga* using PTC-F and PTC-R primers. PTC-1 fragment was used as probe.

B. palonga is a rosette, annual leafy vegetable having two distinct life phases, vegetative and reproductive. Vegetative development is characterized by the production of rosette leaves with little internode elongation. During reproductive phase the plant produces a elongated rachis with spikelet-type of inflorescence. SEM and histological examination of the normal and mutant inflorescences revealed that in the mutant, the normally undifferentiated terminal region of the inflorescence meristem gave rise to floral organs. These features are macro-morphologically similar to the terminal flower mutant of *Arabidopsis*[4,6,8].

B. palonga is a long day plant, but the mutant flowers early and completes its life cycle faster than the control. Stagger plantation experiment revealed that the mutant bolts irrespective of the date of showing, day length and temperature, indicating its day neutral nature (Figure 2). Incidentally terminal flower mutant phenotype of *Beta* (day neutral plant) shows a pattern similar to *Arabidopsis TFL1* mutant[21]. Three *Arabidopsis* genes that control flowering time have been isolated: *LFY*, *AP1* and *TFL1*. In transgenic *Arabidopsis*, over-expression of *LFY*/*AP1* or down-regulation of *TFL1* shortens the juvenile period and causes early flowering[22,23], whereas over-expression of *TFL1* causes late flowering[11].

X-ray induced mutation frequently causes major cytological abnormalities, such as chromosomal deletion or duplication. Most of the cytologically abnormal mutants are unstable and show major morphological abnormality[24]. But in some cases it causes point mutation or induces internal transposon and produces a stable mutant like *CEN* mutation of Snapdragon[20]. Therefore, a karyotype analysis was undertaken to investigate the material cytologically. The karyotype analysis of normal and mutant of *Beta* ($2n = 18$) showed no cytological difference (data not shown). The previous results published from our laboratory[3] indicate that the phenotypic alteration resulted due to mutation of a single gene. This type of phenotype was also observed in other plants[4,6,9,20].

From this study it appears that either there is a mutation of the homologue of *TFL1*/*CEN*-like gene in the mutant *B. palonga*, or the involvement of another gene *FT* which along with *TFL* acts as a activator/suppressor of flowering as in *Arabidopsis*[25].

To explore the first possibility, the degenerated primers were designed from the highly conserved sequence region of the *TFL1/CEN* genes from diverse plant species. The degenerated primers amplified finally to 238 base pair-specific cDNA product, which was renamed as *PTC-1* (*PTC*: Palong *TFL1*/*CEN*-like gene).

For predicting the homology, a BLASTX 2.2.10 program was run using the sequence information of *PTC-1*. The results showed that *PTC* has above 65% identity with *TFL1*/*CEN*-like genes isolated from different plants (Table 2). BLASTX results indicate that a high degree of homology exists with the already identified TFL1-like proteins, with an *E*-value ranging from 0.0030 to 0.0050. Thus the result re-establishes the fact that TFL1/CEN-like protein is taxonomically conserved among diverse plant species, including *B. palonga* and is expressed during inflorescence development[18,25–29].

To determine the evolutionary relationships between PTC and other TFL1/CEN-like proteins, a phylogenetic tree was constructed using the predicted protein sequences (79 amino acids) which were used in multiple alignment. The generated phylogram is represented in Figure 4 which is consistent with the currently accepted species phylogenies[30].

A Southern blot experiment was done using *PTC-1* fragment as probe and the genomic DNA was digested using three different hexacutters, *Eco*RI, *Hin*dIII and *Eco*RV, on the basis of the virtual digestion result of *PTC-1* linear sequence. As shown in Figure 5, two bands were detected in case of normal where DNA was digested with *Eco*RI and *Eco*RV, but with the same restriction enzymes, the mutant showed a single band. On the other hand, *Hin*dIII digested DNA of normal and mutant showed a single ~1.9 kb band in both cases. Thus the Southern result detected the alteration in genomic sequence of one of the homologues of the *TFL1*-like sequence present in mutant *B. palonga*. However, it is difficult to explain non-detection of the altered *TFL1* homologue in the mutant through Southern hybridization. A similar type of result has also been obtained by Cremer *et al.*[20], where the X-ray mutation of *Antirrhinum* resulted in mutation followed by transposon insertion within the *CEN*.

PCR amplification of control and mutant genomic DNA with PTC-F and PTC-R and reconfirmation with Southern blot hybridization revealed that there was a definite alteration of the TFL1 homologue present in mutant *B. palonga*, which was defined as PTC-1. The sequence results of the amplified bands indicated that there were two TFL1 homologues present in *B. palonga*, in which one was not amplified in the mutant. This result indicates that there are two TFL homologues, PTC-1 and PTC-2, of which PTC-1 is affected in mutant *B. palonga* probably due to an excision.

Significance

The terminal flower mutant of *B. palonga* passes through its life cycle rapidly, but produces relatively small amount of seeds. In contrast, normal *B. palonga* grows much longer and eventually produces much large quantity of seeds. In the same way, some plants have a short lifespan; whereas others such as trees, accumulate substantial reserves over many years before reproducing. It remains to be seen whether up or down regulation of *TFL1*-like gene expression through transgenic technologies could induce or reduce the vegetative phase of diverse crop plants. It will, therefore, be interesting to assess how *TFL1*/*CEN*-like genes function in diverse plant species; the present investigation has paved the way for this.

Production of terminal flower is considered to be an ancestral state from which the indeterminate condition evolves[31]. By accelerating progression through phases, the terminal flower mutant, in effect, recapitulates the ancestral form. The evolution of an indeterminate inflorescence from an ancestor with a determinate inflorescence might be regarded as an example of neoteny[32], a process in which juvenile traits persist into later periods of the life cycle. According to this view, the indeterminate growth of the wild-type apex may have arisen from *TFL1/CEN* or its homologue activity, retarding its progression and ensuring that it never reaches the mature determinate floral phase, exhibited by the ancestor[11]. *TFL1/CEN* and its homologues are closely related and belong to a small gene family (*CETS*) that encodes ~23 kDa proteins[33]. Thus genomic information of *TFL1/CEN* homologue from different plant species plays a major role in the construction of phylogenetic relationship among diverse flowering plants.

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