

RESEARCH ARTICLE**Characterization and in silico analyses of RTCS gene from sugarcane encoding LOB protein family of transcription factors: a key regulator of shoot-borne root initiation**

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Abstract

RTCS (Rootless concerning Crown and Seminal roots) is a member of the plant-specific LATERAL ORGAN BOUNDARIES (LOB) domain (LBD) protein family of transcription factors. The RTCS gene has been identified as a key regulator of shoot borne root initiation in maize. In this study an attempt has been made to characterize RTCS from a wild related genus of sugarcane *Erianthus arundinaceus* and compared to the RTCS gene isolated from commercial sugarcane hybrid genotypes. The candidate gene has been successfully isolated from two genotypes of *E. arundinaceus* (SES 288 and IND 04-1335: *EaRTCS*) and two commercial sugarcane hybrid genotypes (Co 775 and Co 86032: *ShRTCS*). The isolated RTCS full length gene showed significant variation between *EaRTCS* and *ShRTCS* at the DNA and protein level. The RTCS amino acid sequence of all the four genotypes indicated the presence of the characteristic structural features of LBD proteins such as a C-motif, LOB domain sequence and leucine zipper-like dimerization domain. Prediction and analysis of secondary structure of RTCS protein in SES 288 and IND 04-1335 showed the presence of 65% alpha helix, 1% β strand and 38% disorder, while sugarcane commercial genotypes showed 58 % alpha helix, 1% β strand and 47% disorder. Both the sequences of *EaRTCS* and *ShRTCS* was found to be closely related to *Zea mays* RTCS.

Keywords: Sugarcane; Root; Drought; Germplasm; *Erianthus*

Introduction

Globally water is becoming a shrinking resource particularly for agricultural purpose. Along with water shortage increasing frequency of drought worldwide leads to crop failures (Wang et al. 2003; Lobell et al. 2011; Lakshmanan and Robinson 2014). Sugarcane is an economically important crop for sugar in the world and it is cultivated in 5 mha in tropical and subtropical India. It is usually planted during December-February months in tropical India. As an annual month crop it undergoes moisture as well as high temperature stress during summer (March to May) which is the critical formative or tillering phase

for the crop. Moisture stress along with high day temperature is reported to cause poor growth, reduced internode length, reduced tillering and high tiller mortality, all put together can lead up to 60% lose in sugarcane productivity (Basnayake et al. 2012; Gentile et al. 2015; Hemaprabha et al. 2013; Hemaprabha et al. 2004).

Among abiotic stresses drought is one of the major constraint affecting agriculture production specifically under rainfed conditions. Plant roots utilize trait specific plasticity to adapt to and respond to the soil conditions. Identifying those specific root traits that increase the capacity of root foraging and sustain productivity is essential

for targeted crop improvement under resource limiting condition. To understand the adaptive plasticity of roots in any crop a clear understanding on the root system architecture is essential. Sugarcane root system is reported to be highly divergent with different root types formed during different phases of its development (Smith et al. 2005). A primary root is formed from the root eyes of vegetative setts during the germination of buds. These sett roots arise within 24 hours of planting the setts and are essentially required for seedling development and ultimately degrade within 60-90 days (Glover 1967). After 5-7 days of planting stable, thicker, fleshier permanent shoot borne roots arise from the shoot nodes. Shoot borne root system forms the healthy and permanent root system for sugarcane that is required for adult fitness and crop yield (Smith et al. 2005). Shoot borne roots which are the major part of the adult root stock grows deeper through agglomeration of two or more roots called rope roots and are reported to be more climate resilient (Smith et al. 2005; Valarmathi et al. 2018). These roots could be an efficient root system to study drought adaptive plasticity with a view to develop drought tolerant sugarcane genotypes. So far there are no reports on the structural and functional aspects of sugarcane shoot borne root system.

To formulate effective strategies to adaptation under drought tolerance, information on molecular basis of shoot borne roots in response to water stress is essential. Literature survey led to the identification of a gene called RTCS (Rootless concerning Crown and Seminal roots) a member of the plant-specific LATERAL ORGAN BOUNDARIES (LOB) domain (LBD) protein family of transcription factors (Taramino et al. 2007). The RTCS gene has been identified as a key regulator of shoot borne root initiation in maize and *rtcs* mutant has been reported to be devoid

of shoot borne roots in maize (Hetz et al. 1996; Taramino et al. 2007). The shoot borne roots or otherwise called as post embryonic crown roots in maize are essential for lodging resistant. Therefore, lodged maize plants were devoid of shoot borne roots and a mapping population between lodged and non-lodged segregating progenies led to the identification of the RTCS gene. LBD family of proteins are shown to be involved in regulating a variety of developmental processes in *Arabidopsis* (Okushima et al. 2007; Lee et al. 2009). Functional analyses of the RTCS gene indicated that it is involved in auxin-mediated signal transduction. RTCS contains LBD element (5'- GCGGCG-3') in their promoter region and expression of RTCS is activated by binding of auxin response factor called ARF to the LBD elements (Okushima et al. 2007). This ability of DNA binding to activate downstream genes, nuclear localization of RTCS proved the feature of a transcription factor (Majer et al. 2012). The characteristic structural features of LBD proteins the LOB domain of RTCS comprises a C-motif putatively forming a zinc finger responsible for DNA binding, a conserved glycine residue and a possible leucine zipper-like dimerization domain (Taramino et al. 2007).

Preliminary studies on sugarcane root system architecture indicated the difference in shoot borne root initiation and biomass among wild relative of sugarcane *E. arundinaceus* and commercial variety (Valarmathi et al. 2018). Morphological, physiological and gene expression patterns in sugarcane in response to waterlogging stress at seedlings stage were studied earlier (Devi et al. 2018). Based on the literature survey and the preliminary sugarcane root system architecture analyses the present study has been carried out to isolate and characterize RTCS gene from sugarcane which is a key regulator of shoot borne initiation. The candidate gene has been isolated

from two genotypes of a wild relative of sugarcane *E. arundinaceus* and two commercial sugarcane genotypes. The isolated RTCS full length gene was subjected to sequence characterization. The sequence features were compared among *E. arundinaceus* and commercial genotypes at the DNA and Protein level. In silico analyses of the translated RTCS protein were carried out to understand the structural features and genetic relatedness of the protein.

Materials and Methods

Genetic material and genomic DNA isolation

Two Indian germplasm accessions of a wild related genera of sugarcane *E. arundinaceus* (SES 288 and IND 04 1335) and two commercial sugarcane varieties (Co 775, and Co 86032) were used in the study. The germplasm clones were obtained from the field gene bank maintained at ICAR-Sugarcane Breeding Institute, Coimbatore, India.

Healthy leaf samples from each genotype were collected and frozen immediately in ice and further used for DNA isolation. The leaf samples ground to a powder in liquid nitrogen and genomic DNA was extracted using CTAB method (Doyle 1991). The quality and quantity of extracted genomic DNA were analysed by Nanodrop and resolving in 1% (w/v) agarose gel.

Designing of heterologous primer and PCR amplification

To isolate the full-length sugarcane RTCS gene heterologous primers were used. RTCS genes from different species were downloaded from NCBI database and consensus primers were designed across highly conserved regions using primer 3 software. A gradient PCR was set to standardize the annealing temperature for the designed primer pair. The primer sequence and PCR conditions used in the study are given in Table 1 and 2.

Cloning and Sequencing of RTCS gene

Amplified RTCS PCR products were resolved in 1 % agarose gel electrophoresis and the bands corresponding to the expected size were cut out from the gel and eluted using gel extraction kit (M/s. Favorgen gel/PCR purification kit). The eluted products were checked again through agarose gel electrophoresis and used for ligation. The eluted fragment was ligated into pTZ57R/T vector and the ligated product was transformed into competent cells of *E. coli* (XL₁ Blue) using heat shock method. The XL₁ Blue cells transformed with the plasmid DNA were selected on LB agar plates containing ampicillin (100 mg/l) and the presence of RTCS gene was confirmed through PCR. Plasmid DNA was isolated from the positive transformants and subjected to Sanger sequencing.

Table 1. RTCS primer sequences used for PCR amplification

Primers	Sequences
RTCS FP	ATGACGGGGTTCGGGTCACCGTGC
RTCS RP	TTACGAGCGATGGTTCAGGTAAGCGTAAGCCACG

Table 2. PCR conditions used for the amplification of RTCS fragment

Step 1	94°C for 1min	Initial denaturation
Step 2	94°C for 30 sec	Denaturation
Step 3	64°C for 30 sec	Annealing
Step 4	72°C for 30 sec	Extension
Step 2-4 repeated with 30 cycles		

Sequence characterization of RTCS gene

The vector sequences were removed from the RTCS genomic sequence reads. The cleaned nucleotide and the translated protein sequence were subjected to BLASTn/BLASTp search against RNA/cDNA/protein in the NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The conserved/catalytic domains in the deduced amino acid sequence of RTCS was analysed using ExPaSy tool (<http://www.expasy.ch/tools/dna.html>). The secondary structure and disorder prediction of the RTCS protein was carried out using ExPasy (Protein Homology/analogy Recognition Engine V 2.0) Phyre 2 software using hidden Markov models via HHsearch (<http://www.sbg.bio.ic.ac.uk/phyre2>).

Multiple sequence alignment and phylogenetic analyses

Multiple sequence alignment of nucleotide and amino acid sequences of RTCS was carried out using CLUSTALW tool in NPS@CLUSTALW software and used for phylogenetic analysis. The genetic relatedness of sugarcane RTCS isolated from *E. arundinaceus* and commercial genotype were compared to that of 28 other RTCS sequences of different crops retrieved from NCBI. The evolutionary distances were computed using

the Poisson correction method of MEGA7 and tree was constructed using UPGMA method with 1000 bootstrap replicates in MEGA7 (Kumar et al. 2004).

Results and Discussion

The gene RTCS (Rootless concerning Crown and Seminal roots), a member of the plant-specific LATERAL ORGAN BOUNDARIES (LOB) domain (LBD) protein family of transcription factors was first identified from maize and reported to play a key role in the initiation of shoot borne roots (Hetz et al. 1996; Taramino et al. 2007). The maize *rtcs* mutants were devoid of post embryonic shoot borne roots and tend to lodge at the seedling stage itself. These mutants completely depend upon functional primary root or embryonic roots for the development of whole plant (Xu et al. 2015; Majer and Hochholdinger 2011). In *Arabidopsis* several homologs of LBD proteins are reported to be involved in auxin signalling and lateral root emergence (Okushima et al. 2007; Lee et al. 2009). RTCS genes from crops such as maize, sorghum and rice has already been reported (Liu et al. 2005; Inukai et al. 2005; Taramino et al. 2007). Similar to maize, sugarcane exhibits a shoot borne permanent root system, but so far, no RTCS

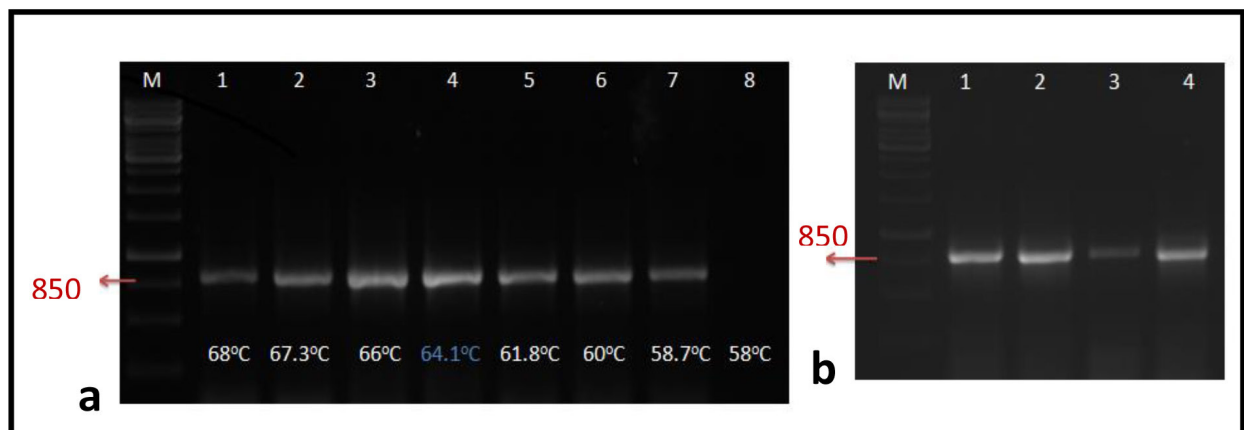


Figure 1. (a) Gradient PCR showing the amplification of RTCS at different temperature, M- 1 Kb ladder, Lane 1-8 indicates different temperature, (b) 850 bp RTCS fragment isolated from four genotypes. M- 1 Kb ladder, 1-SES 288, 2-IND 04-1335, 3- Co 775 and 4- Co 86032

gene has been reported. This study forms the first report on the molecular characterization of RTCS genes from a wild related genera of sugarcane *E.arundinaceus* and commercial sugarcane genotype *Saccharum spp.* hybrids. A single RTCS full length gene fragment of 850 bp was successfully isolated from *E.arundinaceus* (SES 288 and IND 04-1335, designated as *EaRTCS*) and commercial sugarcane genotype *Saccharum spp.* hybrids (Co 775 and Co 86032, designated as *ShRTCS*) using the heterologous primers (Fig.1a and b).

The gene sequence of RTCS isolated from the

four genotypes were analysed for their sequence features. The RTCS genomic sequence of *E. arundinaceus* (SES 288 and IND 04-1335: *EaRTCS*) was 819 bp and the commercial genotype *Saccharum spp.* hybrids (Co 775 and Co 86032: *ShRTCS*) was 792 bp respectively. The full-length genomic sequence of RTCS in all the four samples consisted of two exons separated by one large intron of size 33 bp. The full length cDNA of *EaRTCS* was 780 bp and the commercial genotype *Saccharum spp.* hybrids was 753 bp respectively. The translated *EaRTCS* coded for 260 amino acids and *ShRTCS* coded for 251 amino acids respectively. A full length cDNA of 735 bp with



Figure 2. Multiple sequence alignment of nucleotide sequences of RTCS isolated from *EaRTCS* (SES288 and IND 04-1335) and *ShRTCS* (Co 775 and Co 86032) along with *Zea mays* RTCS (*Zea 23*). Black boxes indicates *EaRTCS* specific nucleotide variation and green boxes indicate *ShRTCS* specific nucleotide variation. The introns are shown in blue font.

two exons separated by a 96-bp intron is reported from maize, rice and Arabidopsis (Inukai et al. 2005; Iwakawa et al. 2002; Liu et al. 2005). The gene structure of *EaRTCS* and *ShRTCS* resembled the structure of other known members of the LOB domain gene family reported from maize, rice and Arabidopsis.

NCBI blast analyses of *EaRTCS* showed 93.21 % homology and *ShRTCS* showed 91.56 % homology to *Zea mays* rootless concerning crown and seminal lateral roots (rtcs) gene, complete cds (EF051735.1) with an E value of 0.0. Both *EaRTCS* and *ShRTCS* sequence showed next maximum homology (89 %) to *Sorghum bicolor* LOB domain-containing protein 6 (XM_002465806.2) mRNA with an E value of 1e-145.

RTCS multiple sequence similarity and in silico analysis

Multiple sequence alignment of *EaRTCS* and *ShRTCS* along with *Zea mays* RTCS showed 87.44% identity, while the translated amino acid sequence showed an identity of 84.04% respectively (Fig. 2 and 3).

The multiple alignment of genomic sequence of *EaRTCS* and *ShRTCS* showed variations at 20 nucleotide positions compared to *Zea mays* sequence (Fig. 2). A six bp insertion and three bp insertion at 284th position and 628th position was found in *Zea mays*, these sequences were not present in both *Erianthus* and *Saccharum* genotypes. There were eleven variations (one eight nucleotide, two three nucleotide, two two

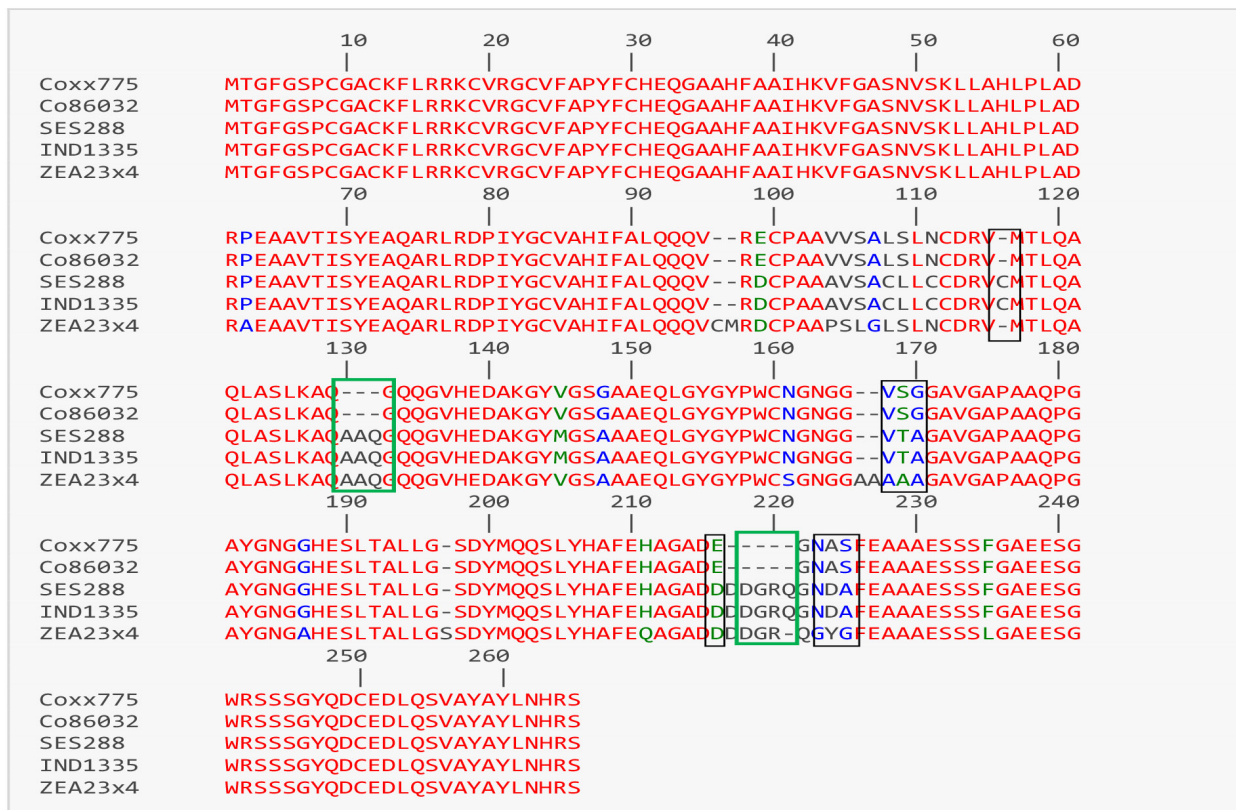


Figure 3. Multiple sequence alignment of amino acid sequences of RTCS isolated from *EaRTCS* (SES288 and IND 04-1335) and *ShRTCS* (Co 775 and Co 86032) along with *Zea mays* RTCS (Zea 23). Black boxes indicates *EaRTCS* specific nucleotide variation and green boxes indicate *ShRTCS* specific nucleotide variation. C-Motif, LOB domain and Leucine-zipper domains are highlighted.

nucleotide and six single nucleotide) specific to the genotypes of *Erianthus* at different position (Fig. 2). A three bp insertion at 367th position was observed only in SES 288 and IND 04-1335. Very few variations were specific to *Saccharum* hybrids among which a 9bp deletion at 421st position and a 15 bp deletion at 684th position were observed. Some of these nucleotide variations were reflected in translated amino acid (Fig. 3). The nucleotide changes from G to A at 393rd position in *Erianthus* showed an insertion of amino acid at 116th position. Single amino acid variations specific to SES 288 and IND 04-1335 were observed at positions 145, 169, 224 and 225. The nine bp deletion and 15 bp deletion in *Saccharum* hybrid clones were reflected at 130th and 216th position in the amino acid sequence. The other variations specific to each genotype are indicated in Fig. 2 and 3.

Transcription factors are reported to carry Nucleotide Localization Signal (NLS) and these NLS sequences are shown to be characterized by short stretches of positively charged amino acids arginine (R) and lysine (K) in their DNA binding region (Taramino et al. 2007; Kalderon et al. 1984). Both the sequences of *EaRTCS* and *ShRTCS* showed the presence of RRK stretch in its C-motif.

Secondary structure of RTCS

The secondary structure prediction of the RTCS protein for all the four genotypes were carried out to understand the protein stability. The major macromolecular building blocks of proteins are generally constituted by Helices and strands (Benner et al. 1997; Morea et al. 1998; Peng et al. 2006). Prediction and analysis of secondary structure of RTCS protein in SES288 and IND04 1335 showed the presence of 65% alpha helix and 1% β strand, while sugarcane commercial genotypes showed 58 % alpha helix and 1 % β strand (Fig.4a-d). The significant variation in

amino acid sequence among *EaRTCS* and *ShRTCS* could be attributed to the structural variation in helices and strands of the protein.

The RTCS amino acid sequence indicated the presence of the characteristic structural features of LBD proteins. RTCS sequence contained a C-motif from 11th to 24th position which is reported to be putatively involved in forming a zinc finger responsible for DNA binding (Majer et al. 2012; Husbands et al. 2007). The amino acid sequence contained the LOB domain sequence from 25th to 80th position and a leucine zipper-like dimerization domain at 81st to 130th position. About 100 amino acids of the LOB domain are reported to be highly conserved between RTCS genes of various species (Taramino et al. 2007). *Erianthus* specific amino acid variations from 104th to 109th position and an amino acid insertion at 116th position were observed in leucine zipper-like domain.

Proteins are reported to carry unstructured regions called intrinsically disordered or disordered. These regions do not show any stable conformation until or unless they are attached to any substrate (Wright and Dyson 2009). Disorder is an important 'building block' for attaining complexity during evolution from unicellular prokaryotes to multicellular eukaryotes (Schlessinger et al. 2009). The levels of disorder among the four samples of *E. arundinaceus* and *Saccharum spp.* were predicted in the secondary structure of RTCS protein. The disorder was 38 % in SES 288 and IND 04-1335, while 47 % disorder was observed in Co 775 and Co 86032. The disorder percentage was observed to be high in both *EaRTCS* and *ShRTCS* sequences. However, a higher disorder percentage in *ShRTCS* indicates a higher protein instability which needs more extensive *in silico* analysis for improved prediction of the structural nature of such proteins using a more specific predicting tools (Holehouse et al. 2017).



Figure 4. Prediction of secondary structure and disorder of RTCS among SES 288 (a), IND 04- 1335 (b), Co 775 (c) and Co 86032 (d) using ExPASy (Protein Homology/analogy Recognition Engine V 2.0) Phyre 2 software (<http://www.sbg.bio.ic.ac.uk/phyre2>).

commercial genotype revealed significant genetic relatedness with other RTCS homolog sequences. Both the sequences of *EaRTCS* and *ShRTCS* formed a separate clade along with *Zea mays* RTCS (A5H451).

The gene sequence of *EaRTCS* and *ShRTCS* formed two smaller clades. The RTCS sequences from wheat formed a separate clade, while rice along with the wild species formed separate cluster. One sequence of RTCS was closely related to sugarcane RTCS, other *Zea mays* sequences formed a separate clade along with *Sorghum bicolor*. After *Zea mays* the second closely related cladogram of sugarcane RTCS was observed to be of wheat RTCS.

Conclusion

In conclusion, the candidate gene RTCS has been successfully isolated from two genotypes of a wild relative of sugarcane *E. arundinaceus* and two commercial sugarcane genotypes. The isolated RTCS full length gene showed significant variation between *EaRTCS* and *ShRTCS* at the DNA and protein level. The RTCS amino acid sequence of all the four genotypes indicated the presence of the characteristic structural features of LBD proteins such as a C-motif, LOB domain sequence and leucine zipper-like dimerization domain. Prediction and analysis of secondary structure of RTCS protein in SES 288 and IND 04-1335 showed the presence of 65 % alpha helix and 1 % β strand, while sugarcane commercial genotypes showed 58 % alpha helix and 1 % β strand. The significant variation in amino acid sequence among *EaRTCS* and *ShRTCS* was reflected in the secondary structure including alpha helix, β strand and disorder %. Both the sequences of *EaRTCS* and *ShRTCS* formed a separate clade along with *Zea mays* RTCS (A5H451). Further gene expression studies of the gene will throw more light on its functional role under drought conditions.

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