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BdRCN4, a *Brachypodium distachyon* TFL1 homologue, is involved in regulation of apical meristem fate

Rodrigo Machado[1] · Sebastián Elias Muchut[2] · Carlos Dezar[4] · Andrea Guadalupe Reutemann[3] · Carlos Agustín Alesso[4] · María Margarita Günthardt[2] · Abelardo Carlos Vegetti[4] · John Vogel[5] · Nora G. Uberti Manassero[2]

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Abstract

In higher plants, the shift from vegetative to reproductive development is governed by complex interplay of internal and external signals. TERMINALFLOWER1 (TFL1) plays a crucial role in the regulation of flowering time and inflorescence architecture in *Arabidopsis thaliana*. This study aimed to explore the function of BdRCN4, a homolog of TFL1 in *Brachypodium distachyon*, through functional analyses in mutant and transgenic plants. The results revealed that overexpression of BdRCN4 in *B. distachyon* leads to an extended vegetative phase and reduced production of spikelets. Similar results were found in *A. thaliana*, where constitutive expression of *BdRCN4* promoted a delay in flowering time, followed by the development of hypervegetative shoots, with no flowers or siliques produced. Our results suggest that BdRCN4 acts as a flowering repressor analogous to TFL1, negatively regulating AP1, but no LFY expression. To further validate this hypothesis, a 35S::LFY-GR co-transformation approach on 35::BdRCN4 lines was performed. Remarkably, *AP1* expression levels and flower formation were restored to normal in co-transformed plants when treated with dexamethasone. Although further molecular studies will be necessary, the evidence in *B. distachyon* support the idea that a balance between LFY and BdRCN4/TFL1 seems to be essential for activating *AP1* expression and initiating floral organ identity gene expression. This study also demonstrates interesting conservation through the molecular pathways that regulate flowering meristem transition and identity across the evolution of monocot and dicot plants.

Key message

BdRCN4 acts as an ortholog of TFL1 in *B. distachyon*, promoting the indeterminate state of meristems and repressing floral transition, by downregulating *AP1* through changes in LFY/BdRCN4(TFL1) ratio.

Keywords BdRCN4 · TFL1 homologs · Inflorescence meristem regulation · *Brachypodium distachyon*

Introduction

Reproduction is an essential function for the persistence of all species. In complex organisms, sexual reproduction plays a fundamental role in reproduction and increasing genetic diversity. In particular, flowering plants have developed a large and diverse set of specialized mechanisms to facilitate sexual reproduction.

Most plant organs develop from the undifferentiated, totipotent cells that make up the meristems. In higher plants, the shoot apical meristem (SAM) is where two opposing processes occur simultaneously: the maintenance and renewal of the stem cell pool; and the differentiation of some stem cells into new organs (e.g. leaves). In addition,

✉ Nora G. Uberti Manassero
nubertimanassero@fca.unl.edu.ar

[1] Instituto Nacional de Tecnología Agropecuaria (INTA), Estación Experimental Concordia, Santa Fe, Argentina

[2] Facultad de Ciencias Agrarias, Universidad Nacional del Litoral, Esperanza, Santa Fe 3080, Argentina

[3] Instituto de Botánica Darwinion– CONICET, San Isidro, Buenos Aires, Argentina

[4] ICiAgro Litoral, FCA, UNL-CONICET, Esperanza, Santa Fe 3080, Argentina

[5] DOE Joint Genome Institute, Walnut Creek, CA 94595, USA

in some plants the SAM transitions into a flower-producing inflorescence meristem that produces reproductive organs (e.g. flowers). The balance between these processes is strictly controlled during plant development, so the location and time of appearance of a new organ is tightly regulated (Barton 2010). This regulation is visible from the beginning of the cellular organization of SAM, where functional areas with different behaviors and cellular identities can be seen (Bowman and Eshed 2000).

Flowering time is regulated by complex exogenous and endogenous signaling pathways controlling or responding to vernalization, photoperiod, circadian clock, ambient temperature, phytohormones, age and autonomous pathways (Fornara et al. 2010; Peer et al. 2021). Despite the wide diversity of factors controlling flowering, all pathways finally converge in a subset of highly conserved genes named floral integrators. In *A. thaliana*, CONSTANT (CO), FLOWERING LOCUS T (FT), SUPPRESSOR OF CONSTANS OVEREXPRESSION 1 (SOC1), LEAFY (LFY) and APETALA1 (AP1) (Simpson and Dean 2002; Peer et al. 2021) have been shown to be responsible for promoting the transition from vegetative to reproductive state. Before the SAM transitions into a reproductive meristem, TFL1 expression is in part responsible for indeterminacy maintenance and vegetative organ formation. Under inductive conditions, CO promotes FT phloem migration from the leaves toward the SAM (Corbesier et al. 2007; Notaguchi et al. 2008) in order to promote flowering. When the transition occurs, TFL1 antagonize FT competing for FD an 14-3-3 protein interaction, until FT/TFL1 rate is high enough to trigger LFY and AP1 expression (Abe et al. 2005; Ahn et al. 2006; Hanano and Goto 2011; Wigge et al. 2005). By this time, the SAM has transitioned into an inflorescence meristem and TFL1 has become an antagonist of AP1 and LFY function. Negative regulation by TFL1 restricts LFY and AP1 expression to the cells in the periphery of the meristem. These peripheral cells then form floral primordia. At the same time, LFY and AP1 repress TFL1 expression and restrict it to the inner cells of the meristem. Here, TFL1 ensures the maintenance of a pool of undifferentiated cells to ensure the indeterminate growth of the inflorescence meristem (Bradley et al. 1996; Ratcliffe et al. 1998; Fornara et al. 2010; Hanano and Goto 2011). The balance between these proteins and their antagonistic roles controls the plant architecture and controls the pattern of vegetative and reproductive organ development (Blümel et al. 2015; Krylova 2020).

The FT and TFL1 gene products belong to the PHOSPHATIDYL ETHANOLAMINE-BINDING PROTEIN (PEBP) family, which is present in all eukaryotes, with three subfamilies identified in angiosperms: FT, MOTHER OF FT AND TFL1 (MFT), and TFL1 like (Chardon and Damerval 2005). Despite over 60% sequence identity between FT and TFL1, the antagonistic function played by FT and TFL1 is determined by a few key amino acids. Point mutations in 5 positions of the FT sequence (Tyr85 in FT exon 2, Glu-109, Trp-138, Gln-140 and Asn-152 in exon 4) transform this floral promoter into a floral repressor similar to TFL1 (Hanzawa et al. 2005; Ahn et al. 2006; Pin et al. 2010; Ho and Weigel 2014). Besides FT and TFL1, other PEBP-like proteins have been identified in *A. thaliana*: MFT, BROTHER OF FT AND TFL1 (BFT), ARABIDOPSIS THALIANA CENTRORADIALIS (ATC), and TWIN SISTER OF FT (TSF). ATC and BFT acts as floral repressors, fine-tuning the initiation of flowering in response to environmental conditions including photoperiod or salt concentration, respectively (Mimida et al. 2001; Yoo et al. 2010; Huang et al. 2012; Ryu et al. 2014). On the other hand, MFT promotes flowering (Yoo et al. 2010) as does TSF, which is almost identical to FT at the amino acid level, but only in short-day conditions (Yamaguchi et al. 2005; D'Aloia et al. 2011).

The strong conservation of PEBP genes allows the identification and functional characterization of a wide range of TFL1 homologs in angiosperms. Consistent with the sequence conservation, the main function of TFL1 in regulating meristem identity, flowering time and inflorescence architecture is highly conserved between angiosperms (for a review, see Wickland and Hanzawa 2015; Krylova 2020 and Supp. Table 1). However, despite this conservation the role of TFL1 homologs in grasses is not fully understood.

Rice plants overexpressing RCN1-2 had a delay in meristem transitions, resulting in a higher number of leaves produced before the transition to flowering, late flowering and denser panicles with a greater number of branches and seeds (Nakagawa et al. 2002). Similar results have also been reported in maize (*Zea mays*) plants with higher levels of ZCN1-6. Meristem maintenance over time delayed flowering time and resulted in more branched and denser tassels (Danilevskaya et al. 2010a). A contrasting phenotype has been described in barley (*Hordeum vulgare*) *hvcen* mutants, with reduced tillering, early flowering, and reduced number of spikelets and kernels per spikelet compared to wild type (Bi et al. 2019). When expressed in *A. thaliana*, grass TFL1 homologs develop a 35 S::TFL1-like phenotype indicating that the grass homologs are functionally equivalent to TFL1 (Ratcliffe et al. 1998b). Ectopic expression of RCN1-2, BoTFL1 from giant bamboo (*Bambusa oldhamii*) and LpTFL1 from ryegrass (*Lolium perenne*) promotes increased rosette leaf number, delayed meristem transition to the reproductive state, increased main axis branching and a general prolongation of the complete life cycle (Jensen et al. 2001; Zhang et al. 2005; Zeng et al. 2015). Consistently, the expression of these homologs could complement the characteristic phenotype of early flowering, defined main axis

and terminal flower displayed by *A. thaliana tfl1* mutants, showing a high degree of functional homology (Jensen et al. 2001; Zhang et al. 2005; Zeng et al. 2015). However, certain functional divergences have also been shown. An exceptional case where *A. thaliana* plants Ubi::LpTFL1 developed hypervigorous shoots that failed to produce flowers before senescence was reported by Jensen et al. (2001).

Lv et al. (2014) identified and studied functionally the homologs of FT in *B. distachyon* but no functional studies of TFL1 homologs had been reported. We constructed a phylogenetic tree that revealed three TFL1 homologs in *B. distachyon*. In this work, we functionally characterized BdRCN4 homologue belongs to RCN4 clade, whose members, excepting for ZCN4 (Danilevskaya et al. 2010b), have not been studied to date. We analyzed the developmental changes that occurred in a *B. distachyon* BdRCN4 gain of function mutant. Significant extension of vegetative phase, delay in heading time and lower spikelet and grain production were observed. Additionally, ectopic and constitutive expression of BdRCN4 in *A. thaliana* resulted in a delay in the transition of vegetative to reproductive meristem, significant extension of vegetative inflorescence phase, hypervegetative shoots and extended life cycle. Although deeper molecular analysis may be performed, our results showed that hypervegetative shoots are caused by a down-regulation of *AP1* and that LFY/BdRCN4(TFL1) rate seems to be determinant to proper *AP1* expression and flower organ formation.

Materials and methods

Phylogenetic analysis

A phylogenetic tree of TFL1-like proteins of grasses was built using sequences belonging to subtribes Bambusoideae, Chloridoideae, Oryzoideae, Panicoideae, and Pooideae. The 98 ingroup sequences, including BdRCN4 (gene model Bradi5g09270 from Phytozome), and the *PEBP* sequences of *Physcomitrium patens* used as outgroup were compiled from EnsemblPlants (plants.ensembl.org), GenBank (ncbi.nlm.nih.gov), Phytozome (phytozome.jgi.doe.gov), and The Oat Genome (avenagenome.org) (Supp. Table 2). Alignments were performed with MUSCLE (Edgar 2004) and the phylogenetic relationships were estimated applying Bayesian inference using MCMC analysis as implemented in MrBayes v.3.1.2 (Huelsenbeck and Ronquist 2001). Parameters of amino acid substitution were selected using the Akaike Information Criterion implemented in jModeltest v.2.1.6 (Darriba et al. 2012). Two analyses, starting from different random trees, were run for two million generations sampling every 1000 to ensure independence of the successive samples. A 50% majority-rule consensus tree was estimated using posterior probability to evaluate nodal support.

Plant material and growth conditions

Bd21-3 seeds of *B. distachyon* and T-DNA mutant JJ13808 were used in this study (Bragg et al. 2012).

A. thaliana tfl1-1 (CS6167) mutant line was obtained from the Arabidopsis Biological Resource Center (ABRC, http://abrc.osu.edu). LFY-GR seeds were provided by Dr. Frank Wellmer (Goslin et al. 2017).

Plants were grown in soil (Klasmann Ts3 substrate) at 20–22 Cº under long-day photoperiod (16 h light/8 h dark) with intensity of approximately 100 µE m^{-2} s^{-1} for *(A) thaliana* and 500 µE m^{-2} s^{-1} for *(B) distachyon*. For selection, plants were grown in Petri dishes containing 0.5X Murashige and Skoog (MS) medium with 0.8% agar with the proper selective agent.

Phenotypic analyses

(A) thaliana phenotyping was carried out on 34 primary transformants (T0) following (Boyes et al. 2001), periodically scoring plant height, number of rosette and cauline leaves, axillary shoots, primary and secondary branches, siliques, and flowering and senescence time. The phenotyping of *(B) distachyon* was carried out on 8 plants per line, following (Derbyshire and Byrne 2013) and periodically scoring plant height, number of leaves, primary and secondary branches, spikelets, and time of inflorescence emergence.

Gene cloning and *A. Thaliana* transformation

The *BdRCN4* coding sequence was amplified with specific primers (forward 5'GGCTCTAGAATGGCTAGGGCACTGGAAC3' and reverse 5'GGCGTCGACTCAGCGGCGGCGTGCAGC3'), digested with *Xba*I/*Sal*I and cloned into a previously modified pBI121 binary vector. The construct was checked by DNA sequencing, introduced into *Agrobacterium tumefaciens* strain LB4404, and used to transform *A. thaliana* Col-0 and *tfl1-1* plants by floral-dip (Clough and Bent 1998). A cassette containing 35SCaMV::BdRCN4::NOS *terminator* was subcloned into pCAMBIA 1302 in *Eco*RI/*Hind*III sites, and the construct was used to transform LFY-GR plants. Transformed plants were selected on the basis of kanamycin or hygromycin resistance and genotyping.

35S::LFY-GR co-transformation experiment

With the aim to analyze the expression of *LFY* and *AP1*, and its role in the hyper-vegetative inflorescence caused by *BdRCN4* high and ectopic expression, an approach in which 35 S::LFY-GR plants (Wagner et al. 1999) were co-transformed with 35 S::BdRCN4 construct was employed. 35 S::LFY-GR, 35 S::LFY-GRx35S::BdRCN4, 35 S::BdRCN4 plants were divided into two groups, and the inflorescences were sprayed every 2 days with a solution containing 10 mM dexamethasone (Sigma-Aldrich), 0.01% (v/v) ethanol, and 0.01% (v/v) Triton X-100 (Sigma-Aldrich), or with an identical mock solution without dexamethasone. After 10 days, we collected the inflorescences and measured *LFY* and *AP1* expression levels. 6 plants were used per line and treatment, and triplicated biological replicates were collected after 10 days of treatment.

RT-qPCR

Total RNA was isolated from *(A) thaliana* and *(B) distachyon* tissue samples using TransZol Up extraction buffer (TransGen Biotech Co.) following the recommended protocol. Total RNA extracts were treated with DNAse. cDNA synthesis was performed following the recommended protocol using total RNA, oligo(dT) primers, and EasyScript reverse transcriptase (all reagents from TransGen Biotech Co.). qPCR was performed using the AriaMx Real Time PCR System (Agilent Tecnologies) system and the TransStart Green qPCR SuperMix (TransGen Biotech Co.). *ACT2* and *ACT8* actin genes (Charrier et al. 2002) or *SamDC* (Hong et al. 2008) were used for normalization in *(A) thaliana* and *(B) distachyon* samples, respectively. Relative transcript levels were calculated based on the ΔΔCt method in three biological replicates.

Scanning electron microscopy

Plant samples were fixed in FAA (formalin: acetic acid: 70% ethanol, 10: 5: 85, v/v), transferred to 70% (v/v) ethanol, and dissected under an SMZ-10 stereomicroscope (Nikon), and dehydrated with a graded ethanol series plus two final changes of 100% acetone. Dehydrated material was critical point dried with CO2 as intermediate fluid using an EMITECH K850 critical point drier. The dried material was coated with gold–palladium and observed and photographed with a FEI QUANTA 200 scanning electron microscope from the Electron Microscopy Service of the Physics Institute of CCT Rosario (Santa Fe, Argentina).

Statistical analyses

Phenotypic data were analyzed by fitting a linear model with treatment as fixed factor. Model parameters were estimated by the generalized least-squares (GLS) procedure using the R statistical software (R Core Team 2023) and nlme package (Pinheiro et al. 2021). Distribution assumptions and homogeneity of variances were checked through analysis of residuals. Heterogeneity of variances was modeled if needed by including a variance function by treatment. Treatment means and standard errors were obtained using the emmeans (Lenth 2023) package.

Results

Phylogeny of *B. distachyon* TFL1-like sequences

TFL1-like sequences of grasses rearrange in three supported clades (Fig. 1). During grass evolution TFL1 proteins split forming a RCN1-3 clade (PP = 1) and a secondary lineage that diverged forming clade RCN2 (PP = 1) and clade RCN4 (PP = 1). BoTFL1 was the only TFL1-like sequence of *Bambusa oldhamii* (Bambusoideae) available in GenBank. The proteins of the other four subfamilies used in this analysis are unequally distributed in each clade. The Oryzoideae and Panicoideae sequences are duplicated in clade RCN1-3. The chloridoid species *Eragrostis curvula* has two paralogs in both clades RCN1-3 and RCN4, and only one in clade RCN2. Within Pooideae, the three paralogs of *B. distachyon* are distributed in each clade (BdRCN1, Bradi4g42400; BdRCN2, Bradi3g44860; BdRCN4, Bradi5g09270). Unlike the rest of Pooideae members, the Triticeae species (*Aegilops tauschii*, *Hordeum vulgare* and *Triticum spp*.) carry single RCN1-3 sequences, duplicated RCN4 sequences that are distributed in two supported subclades, and do not bear RCN2 representatives. Sequence alignment of segments B and C reveal six aminoacidic positions with evident variability (Fig. 1).

BdRCN4 regulates meristem fate in *B. distachyon*

In order to understand the role of BdRCN4 in *B. distachyon* flowering, the T-DNA mutant was analyzed. In this line, the insertion is located at the 3'UTR of Bradi5g09270/BdRCN4 in opposite direction to the gene (Supp. Figure 1), (Bragg et al. 2012). Expression assays using RT-qPCR showed that *BdRCN4* expression level was increased around five-times in mutants in comparison to Bd21-3 control plants (Supp. Figure 1). So that, we consider the T-DNA insertion is generating a gain-of-function mutant, then we decided to rename this genotype as BdRCN4-OE.

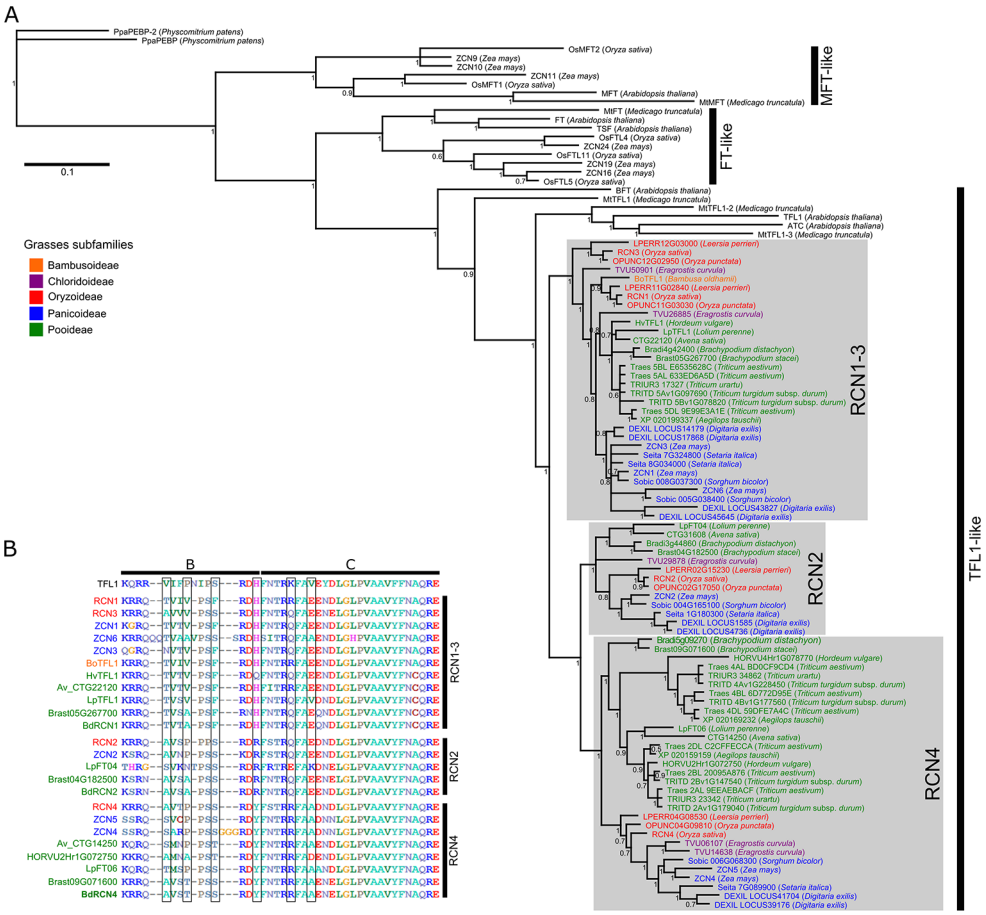

Fig. 1 Phylogenetic relationships of TFL1-like sequences of grasses. (**a**) Phylogenetic reconstruction of TFL1-like protein sequences based on Bayesian methods. Grass sequences are arranged in three supported clades. BdRCN4 (Bradi5g09270) belongs to clade RCN4. (**b**) Alignment of segments B and C of peptide sequences belonging to each clade. Segments B and C are especially important for the determination of functional specificity in TFL1-like sequences (Ahn et al. 2006). Black boxes indicate amino acid positions with appreciable variability

The phenotypical analysis showed no differences in seedlings germination or vigor, but a delay in leaf emergency and development was observed in BdRCN4-OE compared to controls plants (Fig. 2a, d). As shown in Fig. 2d, control plants complete their vegetative growth around 40 days after germination (DAG), whereas BdRCN4-OE plants continued to produce leaves until 60 DAG (Fig, 2b, d). The extension in the vegetative phase allowed these plants to produce more tillers (Fig. 2, d, f), and almost two times as many leaves (35.4 ± 6.7 vs. 66.6 ± 9.0). Consistent with delayed flowering, the transition from vegetative to reproductive SAM occurred around 25 DAG in control plants, whereas BdRCN4-OE meristems remained in a clear vegetative state at 25 DAG (Fig. 2g, h). Interestingly, around half of BdRCN4-OE plants never reached heading time. Flowering individuals showed significant differences in flowering time (34.8 ± 4.8 vs. 85.0 ± 13.7, Fig. 2i) and number of leaves at flowering time (33.0 ± 8.6 vs. 66.6 ± 9.0) compared to control plants, both parameters indicating a strong delay in flowering time. Besides, BdRCN4-OE plants that flowered showed a reduced number of spikes and spikelets (Fig. 2j). A comparative scheme between control and BdRCN4-OE plants is shown in Supp. Figure 1.

Expression of BdRCN4 extends life cycle and prevents flowering in *A. Thaliana*

The 35SCaMV promoter was used to drive the expression of *BdRCN4* in *A. thaliana*. The phenotype of 35S::BdRCN4 was similar to the phenotype of 35S::TFL1 plants (Ratcliffe et al. 1998), except that most of the plants failed to produce flowers and siliques. Seeds of the few independent events that were fertile were collected, but the T2 plants showed no phenotype, probably due to a silencing mechanism (Schubert et al. 2004). Given this peculiarity, we decided to analyze the phenotype in T1 hemizygous plants. 34 independent events were evaluated.

As observed in *B. distachyon* BdRCN4-OE plants, no differences were observed during the vegetative phase of 35S::BdRCN4 plants. However, the transition to the reproductive phase was significantly delayed in *A. thaliana*. 35S::BdRCN4 plants flowered a week later (33.4 ± 2.3 vs. 41.3 ± 2.7) and with twice as many leaves as control plants (16.9 ± 4.3 vs. 39.5 ± 9.7, Fig. 3a, b). When control plants reached the reproductive state, the transition from SAM to an inflorescence meristem was easily recognizable by bolt formation followed by stem elongation. In contrast, most of the 35S::BdRCN4 plants had elongated stems but failed to

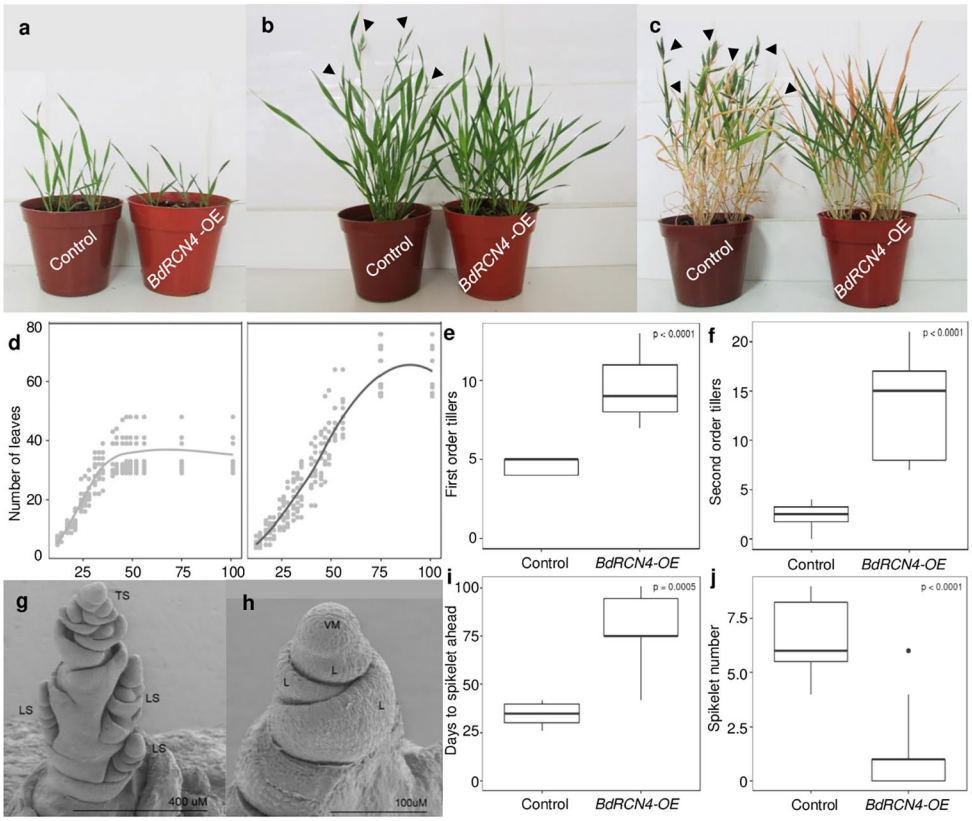

Fig. 2 Phenotypic analysis of *B. distachyon* lines. Control 21.3 and BdRCN4-OE plants at (**a**) 14, (**b**) 33 and (**c**) 56 DAG. Black arrows indicate spikes. (**d**) Plant growth curves. (**e**) First and second order tiller production plots. Scanning electron microscopy show apical meristems from (**g**) WT and (**h**) BdRCN4-OE plants. (**i**) Flowering time and (**j**) spikelet production plots. Means are statistically significant at $p < 0.05$

produce flowers. Bolts were replaced by leafy-like structures (Fig. 3c, d) forming a structure known as hyper-vegetative shoot (Lee et al. 2019). The apical meristem conserved the typical inflorescence symmetry (Fig. 3e, f), but all floral organs were replaced by bracts (Fig. 3g, h). The indeterminacy of the inflorescence was maintained throughout an extended life cycle, generating plants with a longer main axis and a dramatic production of cauline leaves (Fig. 3i). Secondary branches on the main axis were highly increased in 35S::BdRCN4 plants (Fig. 3j), whereas the number of axillary inflorescences diminished (3 ± 1.6 vs. 1.6 ± 1.5). Completion of the life cycle, as measured by full senescence, was delayed (Fig. 3k) and no flowers or siliques were produced by most of the 35S::BdRCN4 plants (Fig. 3l).

BdRCN4 rescue the *tfl1* mutant phenotype in *A. Thaliana*

To further address functional conservation between BdRCN4 and TFL1, *tfl1-1 A. thaliana* mutant was transformed with the 35S::BdRCN4 construct. *tfl1-1* plants exhibited early flowering, reduced plant height, increased axillary shoots and a determinate inflorescence (Shannon and Meeks-Wagner 1991). The *tfl1-1* plants containing 35S::BdRCN4 displayed the same phenotype as wild-type plants containing 35 S::BdRCN4. As shown in Fig. 4, extended vegetative phase and hyper-vegetative shoots were observed. Also, axillary inflorescence development was abolished, and no flowers or siliques were formed in any evaluated plants at 90 DAG. Thus, BdRCN4 seems to be sufficient to rescue early flowering and determine phenotype *tfl1-1* with the indeterminate vegetative state being caused by the constitutive expression.

Hyper-vegetative shoot is caused by AP1 downregulation

In order to find a molecular explanation for the development of a hyper-vegetative shoot instead of flowers and siliques in 35S::BdRCN4 *A. thaliana* plants, we analyzed the expression of *LFY* and *AP1* (Ratcliffe et al. 1998) and transformed 35S::LFY-GR plants (Wagner et al. 1999) with the 35S::BdRCN4 construct. When treated with dexamethasone, 35S::LFY-GR x 35S::BdRCN4 plants developed normal inflorescence and flowers instead of the leafy-like structures, whereas control plants continued to develop bracts (Fig. 5A). Inflorescences were collected and *LFY* and *AP1* expression levels were measured. As expected, *LFY* expression was high in 35S::LFY-GR backgrounds, and were dramatically increased after dexamethasone treatment. Interestingly, no differences were observed between control and 35S::BdRCN4 plants in any condition, indicating that

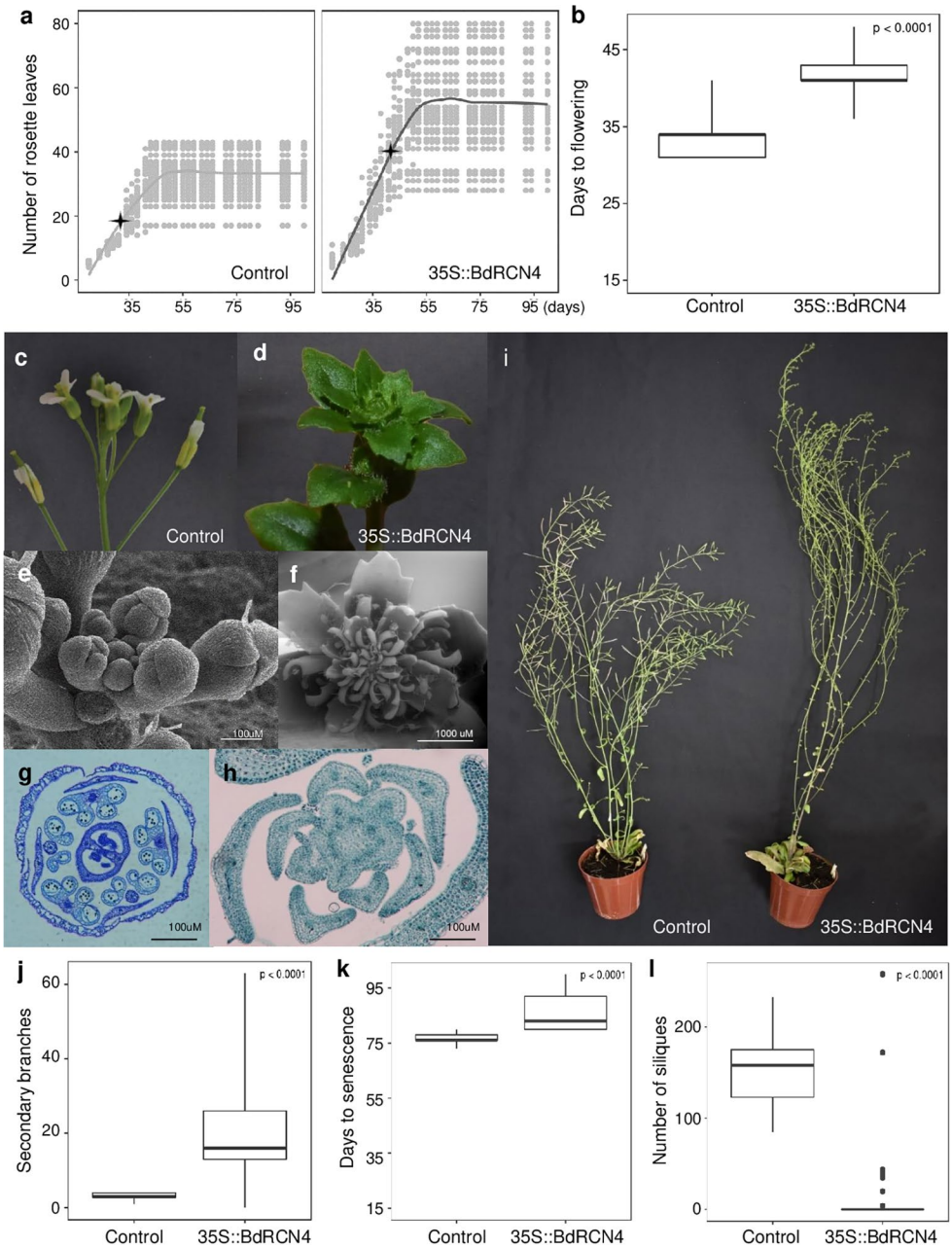

Fig. 3 Phenotypic analysis of Developing differences between 35S::BdRCN4 and control *A. thaliana* plants lines. (**a**) Plant growth curve and (**b**) flowering time plot. (**c, d**) Apical inflorescence of control and transgenic plants (**e, f**) MEB images of inflorescence apical meristems, control and transgenic plants. (**g, h**) Development of flowers and leaf-like structures from control and transgenic plants. (**i**) 33-day-old *A. thaliana* plants. (**j**) Secondary branch production plot. (**k**) Days to complete senescence and (**l**) number of siliques at complete senescence plots. Means are statistically significant at $p < 0.05$

BdRCN4 was not able to regulate *LFY* at the transcriptional level (Fig. 5B). On the other hand, *AP1* was significantly down-regulated in 35S::BdRCN4 plants, both in wild type and 35S::LFY-GR background. After the treatment, *AP1* levels returned to normal in 35S::LFY-GR x 35S::BdRCN4 inflorescences, whereas they remained unaltered in mock and control plants. This *AP1* upregulation is coincident with the formation of inflorescence meristem in 35S::LFY-GR x 35S::BdRCN4 plants leading to the production of normal flowers (Fig. 5A, C).

Discussion

BdRCN4 negatively regulates transition to a reproductive state in *B. distachyon*

This work is an attempt to clarify the molecular pathways that regulate flowering in grasses. *B. distachyon* is an established model for temperate annual grasses including temperate cereals and forage grasses (Draper et al. 2001).

The homologs of PEBP family proteins had been identified in *B. distachyon* by Wei et al. 2014, but no functional studies had been reported for TFL1 homologs. Phylogenetic analysis revealed three TFL1 homologs in *B. distachyon* and

Fig. 4 *tfl1-1* mutant rescue with BdRCN4. From left to right, 90 DAG *A. thaliana* non-transgenic, 35S::BdRCN4, *tfl1-1* mutant and 35S::BdRCN4 in *tfl1-1* background plants

showed an unequal distribution of *TFL1-like* genes among different groups of grasses suggesting functional diversification within grasses. Additionally, the sequence alignments of segments B and C (Fig. 1), which in TFL1 are responsible for delaying flowering (Ahn et al. 2006), identify six amino acids that are variability between clades. This variation may make it difficult to predict the function of particular genes without functional characterization.

In this study we functionally characterized *BdRCN4* in *B. distachyon* and in *A. thaliana* to determine its role in meristem transition and flowering. *BdRCN4* belongs to RCN4 clade, whose members, excepting for ZCN4 (Danilevskaya et al. 2010), have not been studied to date. First, we analyzed a *B. distachyon* T-DNA mutant with an insertion in the 3`UTR (Bragg et al. 2012). As expected for an activating tag, the insertion resulted in *BdRCN4* overexpression (Sup. Figure 1). The overexpression of *BdRCN4* in T-DNA mutant plants correlated with an extended vegetative phase and a significant delay in flowering time compared to wild type (Nakagawa et al. 2002; Danilevskaya et al. 2008; Danilevskaya et al. 2010). Similar results were reported in plants constitutively expressing *RCN1/Oscen2*, *RCN2* and *RCN3/Oscen1* in rice (Nakagawa et al. 2002; Zhang et al. 2005) and *ZCN1-6* in maize (Danilevskaya et al. 2010). Moreover, most of the *B. distachyon* T-DNA plants never flowered. No heading plants were also observed when *RCN1/2* in rice or *LpTFL1* in red fescue (*Festuca rubra*) were overexpressed at high levels, while moderate expression caused a delay in heading time, associated with an increase in inflorescence density (Nakagawa et al. 2002; Jensen et al. 2004; Zhang et al. 2005). In maize, higher inflorescence branching and spikelet density were observed in the tassels of plants ectopically expressing *ZCN1-6* (Danilevskaya et al. 2010). Interestingly, inflorescences of *B. distachyon BdRCN4* overexpressing plants showed no increased order or branching. On the contrary, plants that were able to flower developed a solitary primary inflorescence with fewer spikelets and seeds.

Taken together, the data strongly suggest that BdRCN4 is a negative regulator of flowering in *B. distachyon*, a function highly conserved between TFL1 homologs in angiosperms (for a review see Wickland and Hanzawa 2015; Krylova 2020). Nevertheless, the role of BdRCN4 in regulating transition from vegetative to spikelet meristems seems to differ from other grasses orthologs (Nakagawa et al. 2002; Jensen et al. 2004; Zhang et al. 2005; Danilevskaya et al. 2010; Jin et al. 2019) which could probably had contributed to the great diversification observed in the inflorescence architecture of grasses.

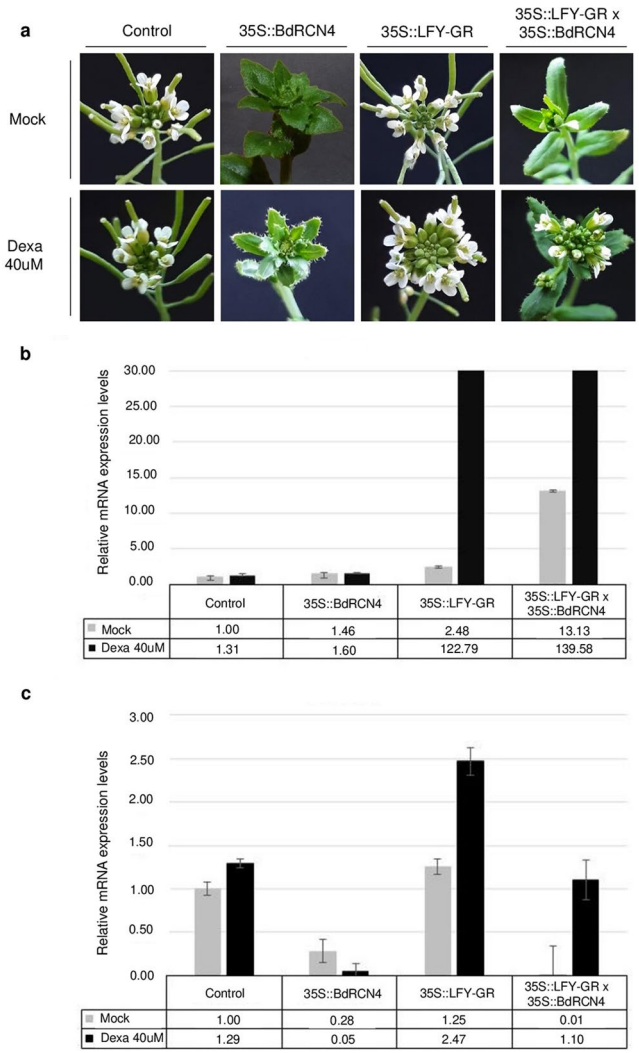

Fig. 5 Analysis of *A. thaliana* 35S::LFY-GR plants transformed with 35S::BdRCN4 plants. (**A**) Inflorescence of *A. thaliana* plants exposed to 40 µm dexamethasone or mock treatment after 10 days. From left to right: Control, 35S::BdRCN4, 35S::LFY-GR, 35S::BdRCN4 in 35S::LFY-GR background. Bar plot of the relative expression of (**B**) *LEAFY* and (**C**) *APETALA1* gene under different treatments and genotypes

BdRCN4 maintains the vegetative state in *A. Thaliana*

In *A. thaliana*, ectopic and constitutive expression of *BdRCN4* caused a delay in flowering time, both in days and number of leaves. Additionally, the inflorescence meristem failed to develop normal flowers, producing hyper-vegetative shoots instead. This phenotype was reported by (Ratcliffe et al. 1998) in 35S::TFL1 plants, and it was cited in most cases when functional studies of TFL1 homologs were carried out using *A. thaliana* (for a review see Wickland and Hanzawa 2015; Krylova 2020; Supp. Table 1). Accordingly, BdRCN4 expression rescued the *A. thaliana tfl1-1* mutant in terms of morphology and phenocopied 35S::BdRCN4 plants, presumably due to the force of the strong 35SCaMV promoter. These results suggest that BdRCN4 is sufficient to supply TFL1 absence, and denote functional conservation in regulating meristem phase transitions, promoting indeterminacy and vegetative state.

BdRCN4 negatively regulates axillary inflorescence meristems

BFT-like genes belong to the TFL1-like clade (Fig. 1), and originated from the duplication of *TFL1/CEN* ancestors in *A. thaliana* (Jin et al. 2021). Sequence alignment and comparison of critical motifs suggested that BFT is more similar to FT than to TFL1, but functional characterization revealed a redundant, even weaker, role as a floral repressor. BFT is unable to complement TFL1 in *tfl1* mutant plants, but modulates plant architecture by negatively regulating axillary inflorescence development (Yoo et al. 2010). In grasses, Jensen et al. (2001) reported that the overexpression of *LpTFL1* in *A. thaliana* led to a significant decrease in the number of axillary inflorescences. We found that, as seen for BFT and LpTFL1, high expression levels of BdRCN4 suppresses axillary inflorescence meristem development (Fig. 3I, J). This is probably associated with an ancient function of *TFL1-like* genes, and the diversification of the roles of these genes for the independent maintenance of the function of apical and axillary meristems. Further studies will be necessary to elucidate if axillary meristem regulation is a function conserved between the three members of TFL1-like proteins in *B. distachyon*, or if, as found for BFT in *A. thaliana* it is specific for BdRCN4.

BdRCN4 suppress flower development

After transition to reproductive state, newly initiated floral meristems arise from the periphery of the inflorescence meristem. The process includes activation of both, inflorescence meristem identity genes such as *TFL1*, and floral meristem identity genes, including *LFY* and *AP1* (Mimida et al. 2001; Benlloch et al. 2007).

TFL1 is an indirect negative regulator of *LFY* and *AP1* expression (Ratcliffe et al. 1998). In addition, it was demonstrated that TFL1-EAR overexpression initially downregulated *LFY* expression, but it gradually started to rise again late in the reproductive state (Hanano and Goto 2011). Nevertheless, the downregulation of *AP1* remained stable in time. Hence, additional components may promote *LFY* expression and countered the downregulation mediated byTFL1, possibly explaining the late flowering phenotype in 35S::TFL1 plants reported by (Ratcliffe et al. 1998).

An interesting pathway was described by Ratcliffe et al. (1998) and Ferrándiz et al. (2000) where they hypothesized that a specific ratio between LFY/TFL1 is necessary for the correct development of floral meristems, and therefore the expression of the floral identity regulators. They observed that combined loss of function mutations in *FRUITFULL* (*FUL*), *AP1* and *CAULIFLOWER* (*CAL*) led to a dramatic non-flowering phenotype, in which plants continuously elaborate hypervegetative shoots in place of flowers. This phenotype was caused both by the lack of *LFY* activation and by the ectopic expression of the *TFL1* gene (Ferrándiz et al. 2000). Additionally, Goslin et al. (2017) reported that when AP1 is absent, LFY promotes *TFL1* expression, decreasing LFY/TFL1 ratio and then inhibiting flower formation.

In our particular case, the hypervegetative shoot phenotype observed in 35S::BdRCN4 plants was similar to that reported by Ratcliffe et al. (1998), with the exception that most of the analyzed plants never flowered. Interestingly, *AP1*, but not *LFY* expression levels, was downregulated. Hanano and Goto (2011) reported that 35S::TFL1-SRDX downregulated *AP1* expression levels throughout the life cycle, but failed to maintain *LFY* downregulation through time. In agreement, Ratcliffe et al. (1998) proposed that *AP1* late upregulation was uncoupled from *LFY* upregulation, suggesting that TFL1 acts more stringently on the pathway governing AP1 activation than on that controlling LFY, mediated by additional factors that were able to overcome TFL1 negative regulation. Considering that 35S::BdRCN4 plants were unable to flower, we imagine two possible scenarios: the negative regulation of BdRCN4 over *AP1* is stronger than that observed for TFL1 or *LFY* expression levels were not enough to reach the LFY/BdRCN4(TFL1) ratio needed to promote flowering.

In order to test the hypothesis, we performed a 35S::LFY-GR co-transformation approach. Interestingly, when 35S::LFY-GR x 35S::BdRCN4 plants were treated with dexamethasone, normal flower formation was restored. As expected, the expression of *LFY* radically increased, followed by *AP1* expression levels raised to normal. These results support the idea that a certain LFY/BdRCN4(TFL1) ratio needs to be reached, in order to effectively activate *AP1* expression. Thus, the activation of floral organ identity genes seems to require a certain threshold of AP1 expression, which only occurs when the TFL1 (BdRCN4) and LFY expression are properly balanced (supported by Goslin et al. (2017).

Several plant species have been analyzed in an effort to understand the role of *TFL1-like* genes and their conservation across angiosperms (as reviewed by Krylova 2020 and Sup. Table 1). However, the function of *TFL1-like* genes in regulating both *LFY* and *AP1* or just one of them remains unclear. In this study, we found that in *A. thaliana*, BdRCN4 regulates floral meristem determination by down-regulating *AP1*, but not *LFY*. Similar results were obtained for ATC and BFT (Yoo et al. 2010). ATC expression is induced under short days and when overexpressed, it produces a phenotype similar to that of 35S::TFL1. However, molecular analyses revealed that ATC interacts with FD to down-regulate *AP1*, but not *LFY* (Huang et al. 2012). Ryu et al. (2014) reported a similar mechanism adopted by BFT in response to high salt stress, where it down-regulates *AP1*, but not *LFY*, through sequestration of FD. Interestingly, 35S::BFT plants displayed down-regulation of both *AP1* and *LFY* gene expression under standard long-day conditions (Yoo et al. 2010). Further efforts are needed to understand the functional overlap between these homologs and their role in regulating floral plasticity in plants.

In conclusion, our study provides convincing evidence about BdRCN4 acts as an ortholog of TFL1 in *B. distachyon*, promoting the indeterminate state of meristems and repressing floral transition. Negative regulation of *AP1* by BdRCN4, acting through delicate changes in LFY/BdRCN4(TFL1) rate, emerged as a key component of this mechanism, although additional investigation is required to elucidate molecular details. Integrating data from different experimental approaches, including protein-protein interaction and gene expression studies, under different environmental conditions, could contribute to a better understanding of the complete mechanism. Further studies in the model grass *B. distachyon* will contribute not only to the basics of flowering regulation but also could have interesting implications in the development of new strategies for flowering management in grasses.

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Code availability Not applicable.

Declarations

Conflict of interest The authors have no conflict of interest to declare.

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