The RING Finger Protein NtRCP1 Is Involved in the Floral Transition in Tobacco (Nicotiana tabacum)

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Received 15 January 2015; revised 23 March 2015; accepted 23 March 2015
Available online 7 April 2015

ABSTRACT

The transition from the vegetative phase to the reproductive phase is a major developmental process in flowering plants. The underlying mechanism controlling this cellular process remains a research focus in the field of plant molecular biology. In the present work, we identified a gene encoding the C3H2C3-type RING finger protein NtRCP1 from tobacco BY-2 cells. Enzymatic analysis demonstrated that NtRCP1 is a functional E3 ubiquitin ligase. In tobacco plants, expression level of NtRCP1 was higher in the reproductive shoot apices than in the vegetative ones. NtRCP1-overexpressing plants underwent a more rapid transition from the vegetative to the reproductive phase and flowered markedly earlier than the wild-type control. Histological analysis revealed that the shoot apical meristem of NtRCP1-overexpressing plants initiated inflorescence primordia precociously compared to the wild-type plant due to accelerated cell division. Overexpression of NtRCP1 in BY-2 suspension cells promoted cell division, which was a consequence of the shortened G2 phase in the cell cycle. Together, our data suggest that NtRCP1 may act as a regulator of the phase transition, possibly through its role in cell cycle regulation, during vegetative/reproductive development in tobacco plant.

KEYWORDS: RING finger protein; Floral transition; Cell division cycle; Tobacco

INTRODUCTION

The transition from vegetative to reproductive growth (the floral transition) is a critical developmental process in flowering plants. During the past two decades, great progress has been made in revealing the molecular mechanisms that regulate the floral transition in higher plants. In Arabidopsis, five major pathways involved in the floral transition have been identified, namely, the photoperiod, vernalization, gibberellin, autonomous, and age pathways (Amasino and Michaels, 2010; Wang, 2014). Various components in these pathways have been well characterized as important regulators of flowering time, including CONSTANS (CO), FLOWERING LOCUS T (FT), SUPPRESSOR OF OVEREXPRESSION OF CON- STANS1 (SOC1), LEAFY (LFY), and FLOWERING LOCUS C (FLC).

The really interesting new gene (RING) family is the largest group of E3 ligases in the ubiquitin-mediated protein degradation pathway. These enzymes participate in multiple plant biological functions, including development, hormone responses, abiotic and biotic stress responses, and cell cycle regulation (Xie et al., 2002; Zhang et al., 2007; Johnson et al., 2012; Luo et al., 2012; Peng et al., 2013; Yu et al., 2013). Several studies demonstrate that RING finger proteins play an important role in the control of photoperiodic flowering...
(Piñeiro and Jarillo, 2013). In Arabidopsis, SINAT5 interacts directly with LHY and inhibits flowering, while COP1, HOS1, RFI2 and DNF negatively regulate flowering time by control the expression level of CO gene (Chen and Ni, 2006; Liu et al., 2008; Morris et al., 2010; Park et al., 2010; Lazaro et al., 2012). In rice, the COP1 ortholog PPS could regulate reproductive-phase change by inhibition expression level of RAP1B, a floral meristem identity protein, resulting in a flowering delay (Tanaka et al., 2011).

The shoot apical meristem (SAM) is a group of stem cells at the tip of the shoot axis, which generate leaves during the vegetative growth stage and flowers in the reproductive growth stage (Poethig 2003; Wang, 2014). During the floral transition stage, an early event observed in many flowering plants is the acceleration of cell division at the SAM (Lyndon and Francis, 1984; Lyndon and Battey, 1985; Bernier, 1988; Francis, 1992; Francis and Herbert, 1993; Jacqmard et al., 2003). Base on this common feature, it has been proposed that an increase in the cell division rate is a prerequisite for the morphological changes that occur at the SAM during the floral transition (Francis, 1992; Bernier, 1997; Jacqmard et al., 2003; Kwiatkowska, 2008). In line with this morphological evidence, flower bud formation occurs earlier in tobacco ectopically expressing Arabidopsis CYCD2 and CYCD3 than the wild type, and the structural organization of the SAM is dramatically altered in these transgenic plants (Boucheron et al., 2005). Although the association of cell cycle regulation with floral induction is well recognized, the molecular basis for the collection between these two processes remains elusive.

In this study, we identified a C3H2C3-type RING finger protein from tobacco BY-2 cells and analyzed the physiological role of this protein in tobacco plants using a reverse genetics strategy. The results reveal that NtRCP1 is involved in the control of the vegetative-reproductive phase transition in tobacco plants, perhaps by regulating the cell division cycle at SAM.

RESULTS

Identification of NtRCP1 from tobacco BY-2 cells and protein structure analysis

In a previous study, we identified a number of cytokinesis-related cDNAs from tobacco BY-2 cells using fission yeast as a functional system (Yu et al., 2007). Ectopic overexpression of one of these cDNAs in yeast cells causes dramatic defects in cell plate formation. As shown in Fig. 1A, the transgenic yeast cells were highly elongated and each contained 2–8 nuclei, demonstrating that overexpression of this plant gene has a strong effect on cell division in yeast.

Sequence analysis indicated that the cDNA contains a 909-bp open reading frame (ORF) and encodes a putative protein with a C3H2C3-type RING finger domain. This protein was designated as NtRCP1 (Nicotiana tabacum RING domain Containing Protein; GenBank accession number: DQ981848) (Fig. 1B). Protein sequence alignment showed that NtRCP1 shares the highest homology with the RING1-like protein of Solanum lycopersicum (Fig. S1).

NtRCP1 possesses E3 ubiquitin ligase activity

The majority of the RING-type proteins function as E3 ubiquitin ligases (Stone et al., 2005). To analyze the E3 ligase activity of NtRCP1, we expressed fusion proteins comprising NtRCP1 and maltose-binding protein (MBP) in Escherichia coli and purified them by MBP affinity chromatography. As shown in Fig. 2, MBP-NtRCP1 fusion proteins catalyzed the formation of a high molecular-weight ubiquitin ladder in the presence of E1 and E2, indicating that NtRCP1 is a functional E3 ubiquitin ligase.

Fig. 1. Identification of NtRCP1 gene and protein sequence analysis.
A: Phenotypes of fission yeast cells harboring empty vector (left) or vector-NtRCP1 (right). Transformed cells were stained with DAPI and calcofluor to visualize nuclei and septa, respectively. B: Amino acid sequence of NtRCP1. The RING domain is shown in the box. Asterisks indicate the conserved cysteine/histidine residues. Bar = 10 μm.

NtRCP1 is involved in the regulation of the vegetative-reproductive transition in tobacco

To investigate the physiological function of NtRCP1 in tobacco plants, we generated NtRCP1-overexpression and RNAi constructs and transformed them into tobacco by Agrobacterium-mediated transformation. The expression levels of NtRCP1 in the transgenic plants were analyzed by RT-PCR (Fig. 3A). Three independent lines (OX 8, 10, and 16) with high NtRCP1 mRNA transcript levels and three independent lines (RNAi 2, 4, and 5) with reduced abundance of NtRCP1 transcripts were chosen for further analysis. Under greenhouse conditions, the growth of both NtRCP1-overexpressing and RNAi transgenic plants were similar to that of wild-type plants in the vegetative growth phase. During the stage of vegetative-reproductive transition, however, the overexpression plants displayed notably faster growth of inflorescence stems and earlier formation of inflorescence meristems (Fig. 3B). As a result, the NtRCP1-overexpressing plants flowered approximately 10-day earlier than the wild-type plants, and a corresponding difference in leaf number was also observed (Fig. 3C and D). The RNAi
plants showed no significant difference to the wild-type plant although a 2- to 4-day delay in flowering time could be observed (Fig. 4C), implying a functional redundancy of NtRCP1 with other homologs in tobacco that can be identified through database search (Fig. S2).

Inflorescence meristems form precociously in NtRCP1-overexpressing plants

The phenotype exhibited by the NtRCP1-overexpressing plants suggests that this gene plays an important role in the initiation of reproductive growth. To assess the function of NtRCP1 at the cellular level, we examined and compared the anatomical structures of the SAMs of wild-type and NtRCP1-overexpressing plants at various developmental stages. At the vegetative stage (20-day-old plants), the SAMs of wild-type and transgenic plants had similar cellular structures with a slight difference of cell number. After 1 month of growth, the SAMs of transgenic plants appeared wider, the cells appeared smaller, the cell layers and numbers were higher (Fig. 4A and Table S1). After 1.5 months of growth, while the SAMs of wild-type plants were enlarging, the shoot apices of transgenic plants had initiated inflorescence (Fig. 4B and C). There results indicate that overexpression of NtRCP1 stimulates cell division at the SAM site, which is correlated with precocious initiation of inflorescence primordia in transgenic tobacco plants.

Analysis of NtRCP1 expression patterns

The qRT-PCR was performed to investigate the expression profile of NtRCP1 in tobacco plants using gene-specific primers (Table S2). As shown in Fig. 5A, NtRCP1 was expressed in all tissues, with the highest expression occurring...
in leaves. As the expression of \textit{NtRCP1} is associated with the transition from vegetative to reproductive growth, we examined the expression levels of \textit{NtRCP1} in the shoot apices of plants at both the vegetative and reproductive stages. The result shows that more \textit{NtRCP1} transcripts accumulated in the shoot apices of plants at the reproductive stage than in those at the vegetative stage (Fig. 5B). This result supports the notion that \textit{NtRCP1} plays a role during the switch from vegetative to reproductive development.

\textbf{NtRCP1 accelerates cell division by shortening the duration of the G2 phase in BY-2 cells}

The presence of an increased number of smaller cells in SAM of \textit{NtRCP1}-overexpressing plants and the formation of multinuclear cells in \textit{NtRCP1} transgenic yeast cells reflected a role of \textit{NtRCP1} in the cell cycle in tobacco. To assess this possibility, we overexpressed \textit{NtRCP1} in BY-2 suspension cells and examined cell cycle-related events. First, we determined the numbers of wild-type and transgenic cells. As shown in Fig. 6A, the \textit{NtRCP1}-overexpressing cells were smaller than the wild-type cells. Additionally, the number of transgenic cells was greater than that of control cells at the stationary phase (Fig. 6B). We monitored cell cycle progression after synchronization of the cells by counting the mitotic index and performing quantitative real-time polymerase chain reaction (qRT-PCR) analysis of \textit{Histone H4} (a marker for S phase) expression. As indicated by \textit{Histone H4} expression, both wild-type and \textit{NtRCP1}-overexpressing cells completed the S phase approximately 3 h after aphidicolin release. However, a striking difference in the duration of the G2 phase was observed between the two types of cells. Using the phase length determination method described by Orchard et al. (2005), the G2 phase in \textit{NtRCP1}-overexpressing cells was determined to be 3 h long, while it was 7 h long in wild-type cells (Fig. 6C and D). Thus, G2 phase was 4 h shorter in \textit{NtRCP1}-overexpressing cells than in wild-type cells. These results indicate that \textit{NtRCP1}...
overexpression increases the cell division rate by reducing the time required for the G2 phase, and resulting in the formation of higher number of smaller cells.

**DISCUSSION**

Growing evidence indicates that RING finger proteins participate in the regulation of flowering time in higher plants. In the long-day plant *Arabidopsis*, the RING finger protein AtRING1A regulates flowering through repressing *MADS AFFECTING FLOWERING* genes, and the E3 ligase HOS1 regulates flowering by mediating CO degradation (Jung et al., 2012; Lazaro et al., 2012; Shen et al., 2014). In this study, we characterized the function of the RING finger protein NtRCP1 in tobacco plants. The results show that increases in *NtRCP1* expression had an effect on the floral transition, suggesting that RING-type E3 ligase also participates in the floral transition in tobacco, a model plant often used to investigate flowering time in day-neutral plants. 

During the floral transition, the size of the SAM increases, with greater numbers of outer layers and higher cell numbers due to shorter cell cycles, which is responsible for floral initiation (Miller and Lyndon, 1976; Bernier, 1988; Nougarède et al., 1991; Jacqmard et al., 2003). Here, we show that increased expression of *NtRCP1* in tobacco plant leads to a precocious transition of SAMs to IMs, which is correlated with enhanced cell division at SAMs. Moreover, we found that overexpression of *NtRCP1* shortened the G2 phase in BY-2 cells. Our results provide a line of evidence showing possible correlation between floral transition and cell cycle regulation at the SAM in tobacco plant.

Spcdc25 is a mitotic activator that is essential for the G2/M transition in fission yeast (Russell and Nurse, 1986). As no ortholog of yeast CDC25 protein was identified in higher plants (Doonan and Kitsios, 2009; Boruc et al., 2010), Lipavská et al., (2011) ectopically expressed the yeast Spcdc25 gene in tobacco to better understand the regulation of the G2/M transition in the plant cell cycle. Spcdc25-overexpressing plants exhibited various phenotypic changes, such as early flowering, altered SAM structure and the formation of a higher number of smaller mitotic cells due to a shortened G2 phase in the cell cycle (Lipavská et al., 2011; Vojvodová et al., 2013). Interestingly, these phenotypes were also exhibited by plants overexpressing *NtRCP1* in the current study. The similarity in phenotypes of tobacco plants overexpressing *NtRCP1* and Spcdc25 implies that like Spcdc25, NtRCP1 may act as a regulator of the G2/M transition during cell cycle progression.

Based on our data, we speculate that in the late vegetative stage, NtRCP1 functions in the ubiquitination and degradation of certain target protein(s) and consequently accelerates cell cycle progression and more interestingly, this function of NtRCP1 is connected with the regulatory network involved in the initiation of inflorescence primordia formation in the SAM. Further studies are needed to identify the target protein(s) of NtRCP1 to unravel the molecular basis for its involvement in the floral transition in tobacco.

**MATERIALS AND METHODS**

**Yeast strain and fluorescence microscopy**

The fission yeast *Schizosaccharomyces pombe* (*h*, *leu1-32*) was used in this study. *S. pombe* was maintained according to standard methods (Alfa et al., 1993). For microscopy, DNA and cell wall materials were visualized using 4′, 6-diamidino-2-phenylindole (DAPI, Roche Diagnostics Hong Kong Ltd., Hong Kong) and calcofluor (Sigma, USA) staining after fixation with 70% ethanol. Photographs were taken using a UV light microscope (Olympus BX51, Tokyo, Japan) with a 100× objective lens.

**Plant material and cell culture**

Tobacco (*Nicotiana tabacum*) cultivar ‘Petit Havana SR1’ was used in this study. Plants were grown under 16 h light/8 h dark conditions at 23°C. Tobacco plant transformation was...
conducted using previously described method (Horsch et al., 1985).

The tobacco BY-2 suspension cells were maintained and transformed according to the method described by An (1985). For cell number determination, stationary cultures were harvested and the same number (1 × 10^7 cells /mL) of wild-type and transgenic cells was transferred to 40 mL fresh medium and grown for 8 days. Samples (0.1 mL) were taken at 24-h intervals and the number of cells per milliliter of culture was counted under a microscope. For mitotic index determination, BY-2 cells were synchronized as described by Nagata and Kumagai (1999). The subcultured cells were harvested every hour and stained with DAPI, and the number of mitotic cells and total number of cells were counted under a UV light microscope. Phase length determination was performed according to Orchard et al. (2005).

Vector construction

The cDNA of NtRCP1 was inserted into binary expression vector pPZP111 (Hajdukiewicz et al., 1994) under the control of the cauliflower mosaic virus (CaMV) 35S promoter to generate the overexpression vector (35S:NtRCP1). For construction of the RNAi vector, NtRCP1 cDNA fragment (approximately 0.5 kb in size) was amplified using primers RNAi-F and RNAi-R (Table S2) and cloned into vector pHANNIBAL in the sense and antisense orientations to create a hairpin structure, and then subcloned into binary expression vector pART27. The recombinant plasmids were respectively introduced into Agrobacterium tumefaciens strain EHA105, and the transgenic strains were subsequently used for tobacco plant or BY-2 cell transformation.

Semi-quantitative RT-PCR and quantitative real-time PCR analysis

Total RNAs were extracted using TRIzol reagent according to the manufacturer’s protocol (Invitrogen, USA). First-strand cDNAs were synthesized from 1 μg of DNase I-treated RNA. One microliter of the first-strand cDNA reaction was used as a template for RT-PCR using LA Taq polymerase (TaKaRa, Japan). The qRT-PCR assays were performed using SYBR Green Real-Time PCR Master Mix (Toyobo, Japan) and a CFX384 real-time PCR detection system (Bio-Rad, USA). The tobacco Actin or Histone H3 gene was used as the internal control. All reactions were performed in triplicate. Primer sequences are listed in Table S2.

Histological analysis

Tobacco shoot apices were fixed overnight at 4°C in a formalin/acetic acid/alcohol fixing solution (50% [v/v] alcohol, 3.7% [v/v] formaldehyde and 5% [v/v] acetic acid). The samples were dehydrated in a series of ethanol solutions (50%–100%) and incubated in 100% ethanol for 1 h, then embedded using a Leica Historesin Embedding Kit (Leica Microsystems, Germany). Serial sections (3 μm thick) of specimens were cut with a rotary microtome (Leica Microsystems), stained with 0.25% (w/v) toluidine blue and viewed under a microscope (Olympus BX51, Japan).

Protein purification and in vitro ubiquitin ligase assays

To express fusion proteins with maltose-binding protein (MBP), the ORF of NtRCP1 was subcloned into expression vector pMAL-c2 (New England Biolabs, USA). Recombinant proteins were purified from E. coli lysozyme according to the manufacture’s instructions.

Ubiquitination assay of MBP-NtRCP1 proteins was performed in the presence of wheat E1, Arabidopsis E2 and ubiquitin (BIOMOL, Plymouth Meeting, USA) in a buffer containing 50 mmol/L Tris—HCl (pH 7.4), 2 mmol/L ATP, 5 mmol/L MgCl2, and 2 mmol/L DTT as described previously. After incubation at 30°C for 2 h, the reaction products were subjected to western blotting and proteins were detected using a mouse anti-ubiquitin monoclonal antibody (Santa Cruz Biotechnology, USA).

ACKNOWLEDGMENTS

We are grateful to Prof. Qi Xie and Dr. Qing-Zhen Zhao (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences) for their kindly help on E3 ligase activity analysis. This work was supported by the Natural Science Foundation of China (Grant Nos. 31100870 and 30800556).

SUPPLEMENTARY DATA

Fig. S1. Sequence alignment of C3H2C3 type RING finger proteins.
Fig. S2. Phylogenetic relationships of NtRCP1 and its homologs in tobacco.
Table S1. Cell layers and numbers in the shoot apical meristem.
Table S2. Primers used in this study.
Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jgg.2015.03.010.

REFERENCES


