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Cellular localization of mitotic RAD21 with repetitive amino acid motifs in *Allium cepa*

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ABSTRACT

Onion can be used in experimental observation of mitotic cell division in plant science because its chromosome is large and easy to observe. However, molecular genetic studies are difficult in onion because of its large genome size, and only limited information of onion genes has been available to date. Here we cloned and characterized an onion homologue of mitotic *RAD21* gene, *AcRAD21-1*, to develop a molecular marker of mitosis. The N-terminal, middle, and C-terminal regions of deduced AcRAD21-1 protein sequence were conserved with *Arabidopsis* SYN4/AtRAD21.3 and rice OsRAD21-1, whereas three characteristic types of repetitive motifs (Repeat-1, Repeat-2/2′, and Repeat-3) were observed between the conserved regions. Such inserted repetitive amino acid sequences enlarge the AcRAD21-1 protein into almost 200 kDa, which belongs to the largest class of plant proteins. Genomic organization of the *AcRAD21-1* locus was also determined, and the possibility of tandem exon duplication in Repeat-2 was revealed. Subsequently, the polyclonal antiserum was raised against the N-terminal region of AcRAD21-1, and purified by affinity chromatography. Immunohistochemical analysis with the purified antibody successfully showed localization of AcRAD21-1 in onion mitosis, suggesting that it can be used as a molecular marker visualizing dynamic movement of cohesin.

1. Introduction

Onion, *Allium cepa* ($2n = 2x = 16$), is not only an agriculturally important plant but also a biologically useful plant for experimental observation of mitotic cell division in plant research (Patau and Swift, 1953) as well as in the academic training of fundamental biology (Gardner and Mertens, 1975; Johnson, 1973). Onion mitotic chromosome (up to 20 μm in size in metaphase) is one of the largest chromosomes in plants, making it easy to observe chromosome behavior in plant mitosis under an optical microscope. In molecular cytogenetic research of onion mitosis, it is important to understand the molecular dynamics of chromosome segregation. Molecular markers, which can visualize the location and dynamic movement of proteins during cell division, are useful for dissection of onion mitosis at a molecular level. Although some antibodies and GFP-fusion proteins from other species can be applied to visualization of onion mitosis, further fundamental information of onion mitotic genes is indispensable to achieve such visualization experiments. However, information of molecular genetics in *Allium* species has been limited so far, and few reports of genomic dissection and sequence analysis of onion genes are available (Jakse et al., 2008; Kuhl et al., 2004; Taylor et al., 2010). To overcome this disadvantage, we have studied onion genes and chromosomes at molecular genetic and epigenetic levels (Do et al., 2001, 2004; Suzuki et al., 2001, 2010a, 2010b). Thus, our aim is to clone onion genes related to mitotic cell division and provide molecular markers to visualize the mitosis (Suzuki et al., 2010a). Here, we attempted to identify a *RAD21* gene in onion, whose product is a major component of cohesin, as a central player of the mitotic cell division.

Cohesin is a protein complex, consisting of SMC1, SMC3, SCC1/RAD21, and SCC3, which is involved in the sister chromatid cohesion during mitosis (reviewed in Losada, 2007; Skibbens, 2009). In this cohesin complex, a heterodimer of SMC1 and SMC3 connected by their hinge domains is closed by association of SCC1/RAD21 αkleisin. Such ring-like structure of the cohesin complex might bundle the sister chromatids from S-phase to mitotic metaphase. The cohesin on chromosome arms and centromeres is dissociated from chromatin until anaphase, permitting the sister chromatids to be distributed to daughter cells. In this step, complete removal of cohesin is achieved by cleavage of SCC1/RAD21 by separase. The fundamental mechanisms of this cohesin-mediated cohesion are conserved and well-studied in yeast and vertebrate. In plants, although the precise biochemical function of cohesin has been largely unknown, molecular genetic studies about orthologs of genes for cohesin components have been mainly reported in *Arabidopsis thaliana* and *Oryza sativa* (da Costa-Nunes et al., 2006; Lam et al., 2005; Schubert et al., 2009; Zhang et al., 2004).

In the case of plant SCC1/RAD21, four *RAD21*-related αkleisin genes, *SYN1/REC8* (*At5g05490*), *SYN2* (*At5g40840*, *AtRAD21.1*), *SYN3* (At3g59550, *AtRAD21.2*), and *SYN4* (*At5g16270*, *AtRAD21.3*), have been reported in *A. thaliana* (Cai et al., 2003; da Costa-Nunes et al.,

Abbreviations: GFP, green fluorescent protein; BAC, bacterial artificial chromosome; ORF, open reading frame.

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2006; Jiang et al., 2007; Schubert et al., 2009; Yuan et al., 2012). In *O. sativa*, *OsRAD21-1* (*Os01g0897800*), *OsRAD21-2* (*Os04g0488100*), *OsRAD21-3* (*Os08g0266700*), and *OsRAD21-4* (*Os05g0580500*) are orthologs of *SYN4*, *SYN2*, *SYN3*, and *SYN1*, respectively (Gong et al., 2011; Tao et al., 2007; Zhang et al., 2004, 2006). *Arabidopsis* SYN1/REC8 plays an essential role in sister chromatid cohesion and chromosome pairing in meiosis (Cai et al., 2003), and rice OsRAD21-4 is also involved in meiotic cohesion (Zhang et al., 2006). On the other hand, *Arabidopsis* SYN4 and rice OsRAD21-1 are predicted to play a major role in a mitotic cohesion (Zhang et al., 2004), although SYN2 and SYN4 might have a redundant function in *Arabidopsis* (da Costa-Nunes et al., 2006). Phylogenetic and functional analyses revealed that SYN1/OsRAD21-4 and SYN4/OsRAD21-1 are major plant αkleisin components of meiotic and mitotic cohesin, respectively, which are also conserved in yeast and vertebrate (Cai et al., 2003; Gong et al., 2011; Zhang et al., 2006), whereas SYN2/OsRAD21-2 and SYN3/OsRAD21-3 might be plant specific and play a distinct role (Gong et al., 2011; Jiang et al., 2007; Tao et al., 2007; Yuan et al., 2012).

In this study, we cloned and characterized an onion *RAD21* gene, *AcRAD21-1* (*A. cepa RAD21-1*), as a potential ortholog of *SYN4*/*OsRAD21-1*. Interestingly, three-types of repetitive amino acid sequences are inserted in AcRAD21-1, resulting in a large RAD21 protein. Localization of the AcRAD21-1 protein during mitosis was consistent with the mitotic RAD21 function, demonstrating that this can be used as a molecular marker in onion mitosis.

2. Materials and methods

2.1. Plant materials

A. cepa (cv. Cheonjudaego, $2n = 2x = 16$) was used for cDNA cloning, BAC screening, sequencing and so on. Other Japanese commercial onion cultivars (cv. Senshu-chukoki and cv. Awaji-chukoki) were also used in immunohistochemical detection.

2.2. Molecular cloning and sequencing

Total RNA was isolated from onion shoots by using the RNeasy Plant Mini Kit (Qiagen). The RNA samples were reverse-transcribed to synthesize first-strand cDNA by using the First-Strand cDNA synthesis kit with a NotI-d(T)18 primer (GE Healthcare). The cDNA was then used as a template for Polymerase Chain Reaction (PCR) amplification with degenerate (RAD21-F2) and NotI-dT-adapter primers (Supplementary Table 1). This reverse transcription (RT)-PCR was performed with *ExTaq* polymerase (Takara), for 35 cycles of denaturation for 45 s at 94 °C, annealing for 45 s at 55 °C, and extension for 1.5 min at 72 °C followed by a final extension for 5 min, by using the DNA thermal cycler TP-650 (Takara). The amplified PCR fragment was cloned by TA Cloning Kit (Invitrogen) and sequenced using 310 Genetic Analyzer (Applied Biosystems). Subsequently, we performed rapid amplification of cDNA ends (RACE)-PCR by using the 5′ RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Invitrogen) with primers listed in Supplementary Table 1 (RAD21-GSP1 to GSP9), and obtained the full-length cDNA sequence. Sequencing of the full-length cDNA was done with RAD21-CSP1 to CSP3 and the RACE primers (Supplementary Table 1). The existence of full-length *AcRAD21-1* transcript was confirmed by RT-PCR with RAD21-NTP and RAD21-GSP1 primers (Supplementary Table 1, Fig. 4a). Nucleotide and amino acid sequences were analyzed by DNASIS software (Hitachi Software Engineering), and the blast search in the DDBJ (http://www.ddbj.nig.ac.jp/searches-j.html).

2.3. BAC screening and sequencing

The partial BAC library of onion (Suzuki et al., 2001) was used for PCR screening (Suzuki et al., 2002) with *AcRAD21-1*-specific primers (RAD21-MPF and RAD21-MPR; Supplementary Table 1). PCR was performed with *ExTaq* polymerase (Takara), for 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 60 °C and extension for 2 min at 72 °C followed by a final extension for 5 min. BAC DNA of a positive clone was purified using QIAfilter Plasmid Midi Kit (Qiagen) and was sequenced by GS FLX system (Roche Diagnostics, Hokkaido System Science Co., Ltd.). The G+C content of genomic sequence was analyzed by GENETIX-MAC Ver. 10.0 software (Software Development Co., LTD.), and gene(s) were predicted by GENSCAN program (http://genes.mit.edu/GENSCAN.html).

2.4. Southern blot analysis

Total DNA was extracted from young leaf tissue by using the DNeasy Plant Mini Kit (Qiagen). Total DNA (about 5 μg) was digested with *Eco*RI or *Hin*dIII (Takara) and separated on 0.8% agarose gel. After electrophoresis, DNA was transferred to nylon membranes (Roche Diagnostics). Hybridization was carried out in 5× SSC, 0.5% blocking reagent (Roche Diagnostics), 0.1% sodium *N*-lauroyl sarcosinate and 0.02% SDS at 65 °C. The membrane was washed twice in 0.1× SSC, 0.1% SDS at 65 °C for 20 min each time. The digoxigenin-labeled probes were prepared by PCR reaction using PCR DIG Labeling Mix (Roche Diagnostics). The detection of the hybridized probe was carried out according to the instructions of the hybridized manual of the DIG Luminescent Detection Kit (Roche Diagnostics) with CSPD as the substrate.

2.5. Antibody preparation

The N-terminal conserved region (N-16) of *AcRAD21-1* cDNA was obtained by RT-PCR with PrimeSTAR HS DNA Polymerase (Takara) using RAD21-NPF and RAD21-NPR primers (Supplementary Table 1). The PCR product was cloned into the pENTR/D-TOPO plasmid (Invitrogen) and re-inserted into pET300/NT-DEST with a 6× histidine (6× His) repeat at N terminal using the Gateway system (Invitrogen). After transformation of BL21 Star (DE3) Oneshot Competent cells (Invitrogen) with the plasmid, the fusion protein was expressed in liquid cell culture using MagicMedia *E. coli* Expression Medium (Invitrogen). Cells were resuspended in 1 ml of 1× PBS(−) (made by using PBS Tablets, Takara; Dulbecco's Formula without Magnesium and Calcium) and lysed by sonication. After centrifugation, pellets resuspended in Binding Buffer (20 mM phosphate, 500 mM NaCl, 20 mM imidazole, 6 M Urea) were purified using the HisTrap FF crude Kit (GE healthcare). A total of 3 mg of the purified ecombinant 6× His-N-16 protein was used to raise polyclonal antisera (PAs-N-16) in two rabbits (Operon Biotechnologies). The polyclonal antibody (PAb-N-16) purified by affinity chromatography was also used for immunostaining.

2.6. Western blot analysis

Young onion shoots were crushed in liquid nitrogen and homogenized with 50 mM Tris–HCl (pH7.5). The protein extract was subjected to SDS polyacrylamide-gel electrophoresis (SDS-PAGE) in the usual manner using the 7.5% PAGE gel (e-PAGEL, ATTO). The fractionated protein in the PAGE gel was electroblotted, and Western blot detection was conducted by using the ECL Western Blotting Analysis System (GE Healthcare) with the rabbit antiserum against N-16 (PAs-N-16) or with the corresponding preimmune serum.

2.7. Immunohistochemical detection

Root tips of *A. cepa* were fixed in 4% paraformaldehyde in PMEG (50 mM PIPES, 5 mM EGTA, 5 mM $MgSO_4$, pH6.8) for 20 min, and treated with 2% pectolyase Y-23 (Kyowa Chemical Products) and 2% cellulase Onozuka-RS (Yakult Honsha) in PMEG at 37 °C for 30 min. After washing with PMEG, cells were squashed on a MAS-coated glass slide (Matsunami), and stocked at −80 °C until used. The