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Characterization of the plant homolog of Nijmegen breakage syndrome 1: Involvement in DNA repair and recombination

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Abstract

The Nbs1 gene is known to code for a protein involved in the hereditary cancer-prone disease, Nijmegen breakage syndrome. This gene is conserved in animals and fungi, but no plant homolog is known. The work reported here describes a homolog of Nbs1 isolated from higher plants. The Nbs1 proteins from both *Arabidopsis thaliana* and *Oryza sativa* are smaller in size than animal or yeast Nbs1, but both contain the conserved Nbs1 domains such as the FHA/BRCT domain, the Mre11-binding domain, and the Atm-interacting domain in orientations similar to what is seen in animal Nbs1. The OsNbs1 protein interacted not only with plant Mre11, but also with animal Mre11. In plants, *OsNbs1* mRNA expression was found to be higher in the shoot apex and young flower, and *AtNbs1* expression increased when plants were exposed to 100 Gy of X-rays. These results suggest that plant Nbs1 could participate in a Rad50/Mre11/Nbs1 complex, and could be essential for the regulation of DNA recombination and DNA damage responses.

Keywords: DNA repair; DNA repair protein; Plant Nbs1; DNA recombination

Mutations in the human *Nbs1* gene lead to Nijmegen breakage syndrome (NBS), a hereditary disorder characterized by microcephaly, chromosomal instability, immunodeficiency, and a high incidence of malignancy. Cells with Nbs1 deficiency were reported to display hypersensitivity to ionizing radiation, decreased homologous recombination [1], accelerated shortening of telomeres [2], and disruption of G1, G2, and intra-S phase checkpoints after exposure to ionizing radiation [3–5]. Nbs1 forms a complex with Rad50 and Mre11, the so-called R/M/N complex, and regulates the catalytic activity and nuclear localization of the complex to insure proper processing and signaling in DNA repair and recombination [6–8].

Plants are much more resistant to ionizing radiation than animals, probably because they contain a higher content of anti-oxidative pigments and are totipotent. Regardless of resistance to ionizing radiation, DNA damage must be properly repaired to maintain the plant genome. Recent achievements in genome sequencing have revealed the existence of plant homologs of many DNA repair proteins, such as the Rad51 paralogs [9,10] and Brca1/Bard1 [11,12]. Regarding the existence of the R/M/N complex in plants, plant homologs of Mre11 and Rad50 have been reported, and AtMre11 is essential for meiotic recombination and DNA repair [13–17]. In contrast to findings for these two subunits of the complex, no Nbs1 homolog was reported to be found in plants. The work described here reports the identification of a plant homolog of Nbs1 in *Oryza sativa* (*OsNbs1*) and in *Arabidopsis thaliana* (*AtNbs1*) using genomic sequence analysis, and the results of tests of the ability of these homologs to function as *bona fide* homologs of mammalian Nbs1.

Materials and methods

Plant material and preparation. Japonica rice (*O. sativa*) and *A. thaliana* (wild type, Colombia) were used as mRNA sources. Rice plants were grown outdoors, and *Arabidopsis* was cultivated in a growth cabinet under a 16 h day/8-h night light regime at 25 °C. Total RNA was isolated from

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plant tissue using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocols.

Plasmid construction. Nbs1 vectors were constructed as described previously [18]. Briefly, cDNAs were synthesized from total RNA using MML-V reverse transcriptase (Invitrogen) and oligo-dT primers. Complete ORF sequences for the desired genes were amplified with PCR using Pyrobest DNA polymerase (TaKaRa). A restriction enzyme recognition sequence for *Nco*I, or *Bam*HI was added to the 5′-end of the *OsNBS1* and *OsMre11* primers for cloning into two-hybrid vectors. The primer sequences used were as follows:

OsNbs1 (forward: 5′-AATAGCCATGGAGGAAGTGTACGA GATGGTGTG-3′; reverse: 5′-AATAGCCATGGAACAAAGGGTCAG GTAGAGGA-3′).

OsMre11 (forward: 5′-TCTGGGATCCACATGCAGGGAGACG AAAGC-3′; reverse: 5′-TAATGGATCCTCATGTCCTCCTAACAG CTC-3′).

AtNbs1 (forward, AtNb2018F: 5′-AATGGTTTGGGGTCTCTTTC-3′, reverse, AtNb4857R: 5′-CCTTAAAACCTCAACTTCCAG-3′).

Primer sequences for human or chicken Nbs1/Mre11 were described previously [18].

PCR products were kinased and ligated into a pBluescriptII vector (Stratagene), and the entire cDNA insert was verified by sequencing. The *OsNbs1* or *OsMre11* cDNA was then subcloned into pAS2-1 (Clontech) or into the GAL4-activating domain of pACT2 (Clontech) for construction of yeast two-hybrid vectors.

Yeast two-hybrid analysis. Full length *OsNbs1* cDNA was expressed as a fusion protein with a GAL4-DNA-binding domain (BD) from pAS2-1 (Clontech) or to a GAL4-activating domain (AD) from pACT2 (Clontech). The full length *OsNbs1*-BD (or -AD) plasmid was transfected into the yeast strain GC-1945 (Clontech) along with a full length *OsMRE11*-AD (or -BD) plasmid. Interaction between the expressed proteins was detected by growth on an SD (Leu-/Trp-/His-) plate and by β-galactosidase activity.

Expression analysis. Rice plant tissue was prepared by cutting root and shoot apex tissue from young plants (14 days or less), flower shoots, and stamens from 60 days old plants. Whole *A. thaliana* plants were exposed to 70 kvp X-rays using an OM-B205 soft X-ray generator (OHMiC, Japan), and were then used as a RNA source. Semi-quantitative RT-PCR was performed to analyze mRNA expression of *OsNbs1* or *AtNbs1*. *Actin1* was used as an internal expression control. The primers used for *Nbs1* are the same as the ones used for vector construction. Primer sets for *Actin1* are as follows: *OsActin1* (forward: 5′-GTATGGTCAAGGCTGGGTTC-3′; reverse: 5′-GTGCACAATGGATGGGTCAG-3′) and *AtActin1* (forward: 5′-AAAATGGCTGATGGTGAAGAC-3′; reverse: 5′-AACAATCG ATGGACCTGACTC-3′).

PCR cycles were optimized to obtain quantitative yields between 18 and 25 cycles, and the amount of template cDNA used for each sample was normalized by using the *Actin1* signal. After electrophoresis, fluorescence intensity was analyzed by Image analysis software (Fuji Film) and the relative expression levels of *OsNbs1* or *AtNbs1* were calculated.

Results and discussion

Identification of plant Nbs1 homologs

The GenBank database was searched for a rice homolog of *Nbs1* using the Blast program which is available at the NCBI homepage (http://ncbi.nlm.nih.gov/). A 30 amino acid sequence, including the Mre11-binding domain from chicken Nbs1 [18], was used as a query. The predicted cell cycle regulating protein (cDNA clone J023022F23, GenBank Accession No. AK069561) was found to contain a conserved Mre11-binding domain, and further analysis by CD-search (conserved-domain search on the Blast program) revealed that the protein also has FHA and BRCT domains at its N-terminus region. The sequence at the FHA/BRCT domain shows a 24% identity with human Nbs1. The consensus sequence for the Atm-interacting region [19] was also found just downstream of Mre11-binding domain (Fig. 1). Next, using the amino acid sequence of OsNbs1, the genomic sequence database of *A. thaliana* was screened with the NCBI BlastX program. A similar FHA/BRCT sequence and Mre11-binding domain was found in the genomic sequence of chromosome III (BAC clone F16B3, GenBank Accession No. ATAC021640). A 10 kb sequence containing the predicted FHA/BRCT- and Mre11-binding domains was obtained from the BAC F16B3 sequence, and these were analyzed with the GENSCAN program (http://genes.mit.edu/GENSCAN.html) in order to predict the entire gene structure. The program predicted that the gene would consist of 11 exons. Several PCR primer sets were then designed for cloning of the entire sequence of the *AtNbs1* mRNA. The AtNB2018F and AtNb4857R primer sets (see Materials and methods) successfully amplified the entire open reading frame of AtNbs1 using RT-PCR. The GenScan program predicted 11 of the 13 exons in *AtNbs1* with almost correct exon/intron junctions. The amino acid sequence of *AtNbs1* showed a 35% identity and 57% similarity with *OsNbs1*.

OsNbs1 and *AtNbs1* consisted of 560 and 533 amino acids, respectively. Their sizes are significantly smaller than mammalian (754 amino acids for hNbs1) or budding yeast Nbs1 (853 amino acids for Xrs2) homologs. However, these two genes do contain the conserved domains from the NBS1 homolog; i.e., the fork-head associated (FHA) domain, and the BRCA1 C-terminus repeated (BRCT) domain at their N-termini and the Mre11-binding domain and Atm-interacting domain at their C-termini (Fig. 1).

Interaction and expression analysis of plant Nbs1

Next, the ability of OsNbs1 to interact with OsMre11, GdMre11 (chicken), or hMre11 (human) was tested using yeast two-hybrid analysis. An apparent interaction between OsMre11 and OsNbs1 was observed (Fig. 2). Interestingly, OsNbs1 could interact with either human Mre11 or chicken Mre11 (Fig. 2). This indicates that the OsNbs1 is a true homolog of mammalian or avian Nbs1.

mRNA expression levels of *OsNbs1* are higher in tissues where frequent cell division or meiosis may occur, i.e., shoot apex and young flower (Fig. 3B), suggesting that plant Nbs1 could be involved in the regulation of cell proliferation or meiosis.

Many plant DNA repair genes are reported to display elevated expression levels after DNA damage, therefore, the expression of *AtNbs1* mRNA was analyzed after exposure to X-rays. An approximately 2.2-fold increase in *AtNbs1* mRNA expression was observed at 3 h after irradiation with 100 Gy (Fig. 3A). This suggests that AtNbs1