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XRCC3, a Target Gene for Termination Technology in Plants

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Abstract

The terminator technology produces seeds not to germinate in the next generation. X-ray cross-complementing group 3 (AtXRCC3) is a protein involved in DNA repair and recombination in Arabidopsis. A knock-out Arabidopsis line for At*XRCC3* inserting T-DNA on a position of 703 bp downstream from the start codon was identified. The homozygous line of At*XRCC3* is morphologically similar to the heterozygous line and wildtype of Arabidopsis until flowering. However the homozygous line did not set seeds in siliques showing sterility. These results suggest that At*XRCC3* is essential for meiosis in plant seed development and is useful as a terminator gene in plants.

Key words: Homozygous; *XRCC3*; Seed development; Terminator.

1. Introduction

The terminator technology which seeds are not able to germinate in the next generation after sowed and cultivated, was developed in kind of control of plant gene expression [1]. Protein synthesis in embryonic development is suppressed by the technology in the action of ribosome inactivation protein under the control of embryo-specific promoter of the LEA gene, which overexpressed in the late stage of embryo development. This technique was improved by the Monsanto company which it produced seeds not to germinate by suppressing the production of the ACOX enzyme which is essential on germination. These kinds of updated technologies are useful to prevent illegal copy of high valued transgenic plants.

DNA repair and recombination mechanisms maintain genetic stability and suppress the formation of aberrant aging events. The damages are occurred not only on single strand of DNA but also on the breaks of double strands (DSB) [2]. The DSB can be generated by artificial methods such as ionization in cells by radioactive rays or treatment of radiomimetic chemicals. There are two well-known repair mechanisms for DSB [3]. One is the non-homologous end joining (NHEJ) and the other is the homologous recombination (HR). Although the repair mechanism of DSB has been revealed to exist in all the living organisms, they differ in which to apply first above two mechanisms. In general, creatures with small genome such as bacteria and yeasts use HR mechanism to repair damages on DSB when similar DNA sequence to the damaged area exists. On the other hands, higher organisms with large genome such as plants and animals uses NHEJ to repair damages on DSB regardless of the existence of similar DNA sequence in a gene to the damaged areas [4]. HR mechanism has been well researched in yeasts and occurs throughout four stages: finding homologous sequence, pairing DNA, strand invasion, and synthesis of DNA to finish repairing [5]. RAD52 epistasis group (RAD50-57, RAD59, MRE11, XRS2) is known as genes contributed to HR repair. These genes play important roles in repairing on DSB and the process of recombination in meiosis. Especially, RAD51 plays central roles in the process of HR as a homologue of RecA recombinase [6]. Several genes related in HR were reported in human based on RAD51 homology. Especially, five RAD51 paralogs (RAD51B/RAD51L1, RAD51C/RAD51L2 and RAD51D/RAD51L3, XRCC2, XRCC3) were reported and there are 20-30% identities between RAD51 and other protein sequences. Moreover, it has been confirmed by yeast two-hybrid assay that RAD51 paralogs interacts with one or more of the others and combine mutually [7]. It has been reported that embryonic lethality occurs by mutations in RAD51 paralogs such as RAD51, RAD51B, RAD51D and XRCC2 [8, 9]. Therefore, it implies that HR repair mechanism plays significant role in human DNA repair. XRCC3 is a DNA repair protein needed to repair DNA damage caused by ionizing radiation or DNA methylating agents. XRCC3 can be combined with DNA and is known to have ATPase activity [10]. The mutations of *XRCC3* induce death in the stage of embryo [9]. Some of these mutations showed higher extent of recombination than the level of occurrence in normal chromosomes indicating that the genes occurring mutations engage in HR or NHEJ. RAD51 and DMC1/LIM15, RecA homologs, have been reported as their engaging in HR in Arabidopsis plants [11, 12]. The researches have been actively conducted in this area with genes related in HR after the determination of whole genome sequences [13].

In this report, we conducted the analysis of a knock-out line for *XRCC3* and elucidated their functions in plants. Here, a gene knock-out of the *XRCC3* which roles on DNA repair and recombination showed two phenotype in their homo-/heterozygous state. The transgenic Arabidopsis inserted T-DNA on *XRCC3* gene on heterozygous showed normal growth and seed production. However, The homozygous insertion on the *XRCC3* gene showed normal vegetative growth until flowering but sterility not to set seeds in siliques. These findings would be helpful to develop a new termination technology not to set seeds using *XRCC3* gene in transgenic crops.

2. Materials and methods

2.1 Plant materials

Arabidopsis plants for wildtype (ecotype Columbia) or transgenic lines from Arabidopsis Biological Resource Center (ABRC) at the Ohio State University were raised in a 22℃ growth chamber with 70% humidity, a light intensity of 100 uE.m-1.s-1 and a photo-period of 16 hours light followed 8 hours darkness in soil (Bioplug, pH 5.5 - 6.5, Seminins Korea) watered with Hoagland 1X solution.

2.2 Multiplex Polymerase chain reaction (PCR)

To isolate homozygous lines from the Arabidopsis lines inserted T-DNA in the gene of *XRCC3*, which is consisted of single exon in Arabidopsis, PCR was performed with the primers indicated below. The plant materials were prepared from leaves of mature plants on soil. *XRCC3* gene was amplified after purifying genomic DNA from the plant materials by DNeasy kit (QIAGEN com.) using primers derived from the genomic sequence of *XRCC3* in GenBank (Acc. No. AB011482): P1 primer (5'-TGGTACCTTTTGGCGGTGAAATG-3') and P3 primer (5'-ATCTCCGGATCCTTCTGCAAT-3'). P2 primer was designed from LB region of T-DNA and additionally added for multiplex PCR to check the homozygous line. P4 primer was designed from RB region of T-DNA and used with P3 primer to check the insertion site of T-DNA in the genome of transgenic plants. The PCR was performed using BiothermTM DNA polymerase(Genecraft Ltd, Munster, Germany) in Perkin-Elmer GeneAmp PCR System 9700(Norwalk, CT). PCR conditions: 5 min at 95°C, followed by 30 cycles: 60 sec at 94°C, 30 sec at 55°C, and 60 sec at 72°C, followed by 7 min at 72°C.

2.3 DNA sequencing and analysis

DNA sequencing was performed by the dideoxynucleotide chain termination method with an automatic sequencer (AlFexpress DNA sequencer, Pharmacia Biotech, Inc.). Nucleotide sequences were compared with sequences present in the GenBank or SIGnAL "T-DNA express" database (http://signal.salk.edu/) in Salk institute and analyzed using BLAST or Biology WorkBench 3.2 (http://workbench.sdsc.edu; San Diego Supercomputer Center; University of California San Diego).

2.4 RT-PCR fallowed by Southern blot hybridization

RT-PCR was performed with the primers indicated below. Total RNA was purified from whole plants by Tri Reagent (Molecular Research Center INC, Cincinnati, Ohio). One μg total RNA was used for reverse transcription in a volume of 25 μl and incubated to 42°C for 1 hour with AMV Reverse transcriptase and oligo d(T) primer (Promega, Madison, WI) and inactivated by incubating 5 min at 95°C. After completion of reverse transcription, 5 μl of RT products was amplified using primers derived from the nucleotide sequence of *XRCC3*: P1 primer and P3 primer (see above in PCR). The PCR was performed using BiothermTM DNA polymerase (Genecraft Ltd, Munster, Germany). PCR condition was the same as described above in PCR section. As a control for RT-PCR, α-tubulin transcripts were amplified with specific primers; TUB2-1(5'-CTCAAGAGGTTCTCAGCAGTA-3'), TUB2-2(5'-CTCAAGAGGTTCTCAGCAGTT-3') for 25 cycles with same conditions described above.

DNA gel blot analysis was carried out to amplify the PCR signals according to a procedure described by Sambrook and Russell [14]. The DNA fragments after RT-PCR were separated by electrophoresis. Followed by denaturation and neutralization, the DNA fragments were transferred to nylon membrane (Hybond-N+, Amersham Pharmacia Biotech. Inc., UK). A radiolabeled probe was prepared from the full-length *XRCC3* cDNA with [α-32P]dCTP, using an oligonucleotide priming kit (RediprimeTM II Random Prime Labeling System, Amersham Pharmacia Biotech. Inc., UK). Hybridization was carried out in 6X SSC buffer with 5X Denhardt´s solution (1% BSA, 1% Ficoll, 1% PVR) and 0.5% (w/v) SDS at 65℃ for 18hr with the radio-labeled probe. Membranes were washed and then exposed to film for autoradiography.

3. Results and Discussion

3.1 Identification of *XRCC3* gene

We previously identified the gene encoding *XRCC3* from Arabidopsis. At*XRCC3* is existed as single copy in Arabidopsis and consisted of single exon containing an open reading frame (ORF) of 915 bp encoded a 304 amino acid polypeptide. The amino acids sequence of XRCC3 are characteristic P-loop for ATP/GTP binding site motif A present in XRCC3 homologs from many animals. The deduced amino acid sequence of AtXRCC3 is highly homologous to the human counterpart.

3.2 Identification of T-DNA insertion site

The SIGnAL "T-DNA express" database (http://signal.salk.edu/) in Salk institute was searched to find out probable knock-out lines of *XRCC3* in Arabidopsis. A seed of Salk_045564 was identified and ordered from ABRC for further analysis. To identify the insertion site of T-DNA on the *XRCC3* gene, the genomic DNA from germinated Salk_045564 plants was purified and PCR was performed with combination of designed *XRCC3*-specific primers (P1 and P3) and LB or RB-specific primers (P2 and P4) (see Materials and Methods). Sequencing of the PCR products revealed that the T-DNA was inserted internal of the *XRCC3* gene on a position of 703 bp downstream from the start codon (Figure 1A).

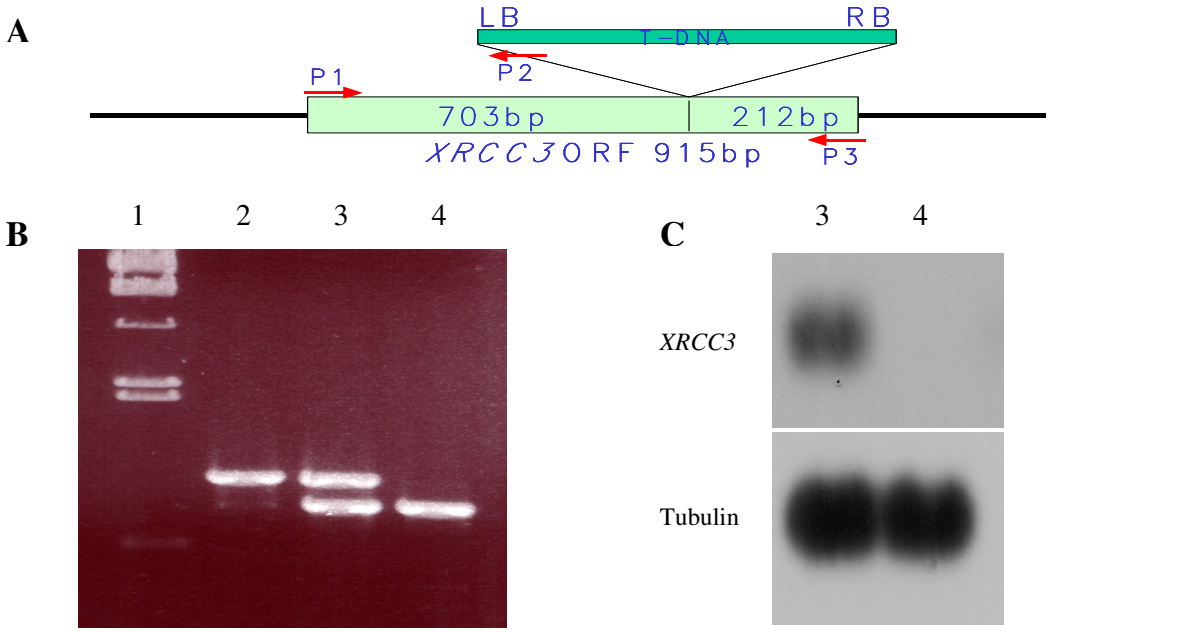


Figure 1. T-DNA insertion site and homozygous line. (**A**): T-DNA insertion site was mapped by PCR followed by DNA sequencing. A schematic drawing of the exact T-DNA insertion site was presented with the region of primers designed. (**B**): Multiplex PCR to identify homozygous lines. Genomic DNA was purified from wildtype (lane 2), heterozygous *XRCC3*+/- (lane 3) and homozygous *XRCC3*-/- (lane 4) plants. Multiplex PCR was performed with three primers. The lane 1 is loaded size marker. (**C**): Northern blot analysis from Arabidopsis plants. The expression of *XRCC3* was tested in the heterozygous *XRCC3*+/- (lane 3) and homozygous *XRCC3*-/- (lane 4) Arabidopsis plants by RT-PCR followed Southern blotting using tublin as loading control.

3.3 Multiplex PCR analysis in the knock-out line of *XRCC3*

To isolate the homozygous line of *XRCC3*, which is derived from the seeds in the T-DNA line (Salk_045564) of Arabidopsis plants, multiplex PCR was performed with P1, P3 and P2 primer sets (see Materials and Methods). Genomic DNA was isolated from leaves of mature plants from wildtype, heterozygous and homozygous Arabidopsis. The expected size of PCR products from three Arabidopsis lines was detected on a gel (Figure 1B). The wildtype Arabidopsis showed single product of 0.9 kb from *XRCC3* gene. However, the heterozygous line showed two PCR products of 0.9 kb and 0.7 kb that was amplified from native *XRCC3* gene and *XRCC3*/T-DNA, respectively. The homozygous line showed only one product of 0.7 kb as expected because it can be amplified from *XRCC3* gene with T-DNA in both alleles. The larger PCR product over 10 kb was not detected in the PCR condition. Our results indicate that we have obtained the heterozygous and homozygous *XRCC3* knock-out lines by segregation from the T-DNA insertion line.

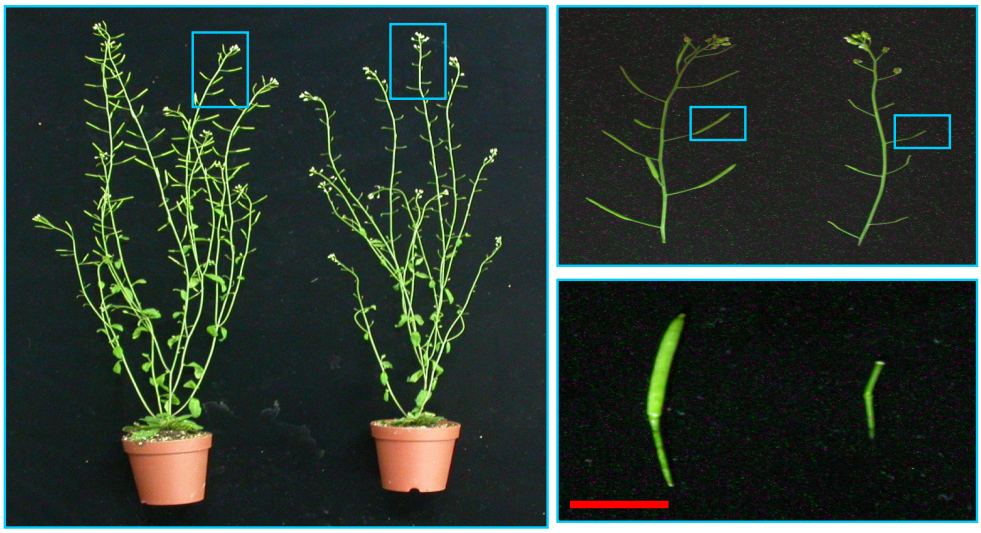
3.4 *XRCC3* expression in the *XRCC3* knock-out lines

To investigate *XRCC3* expression in the *XRCC3* knock-out lines, RT-PCR was performed with designed P1 and P3 primers. Further Southern blot was performed to amplify the signal because it was weak to detect. The expression of *XRCC3* was detected in heterozygous Arabidopsis but completely blocked in the homozygous *XRCC3* line (Figure 1C).

3.5 Morphology analysis in the *XRCC3* knock-out lines

The phenotypic difference in the growth and reproduction was examined in a plant growth chamber. The heterozygous line of At*XRCC3* is normally developed and morphologically similar to

wildtype of Arabidopsis. The homozygous line is morphologically similar to the heterozygous line and wildtype until flowering. However the homozygous line did not set seeds in siliques showing sterility (Figure 2).



XRCC3+/- *XRCC3*-/- *XRCC3*+/- *XRCC3*-/-

Figure 2. Morphology in knock-out Arabidopsis disrupted *XRCC3*. The morphology was examined in whole plants including siliques. The *XRCC3*+/- and *XRCC3*-/- indicate heterozygous and homozygous insertion in the *XRCC3* gene in Arabidopsis, respectively.

We reported that an analysis of a knock-out line of *XRCC3* showed two different reproductive phenotype in their homo/heterozygous state. The transgenic plants inserted T-DNA on *XRCC3* gene on heterozygous state showed normal growth and seed production. However, The homozygous insertion on the *XRCC3* gene showed normal vegetative growth until flowering but sterility not to set seeds in siliques. The knock-out of *XRCC3* is previously reported [15]. Our results reported here, therefore, confirm and extend the first characterization of the *XRCC3* function in Arabidopsis [15]. Our results showed further characterization of *XRCC3* knock-out phenotypes in their homo-/heterozygous state and indicated a possibility that *XRCC3* is a target gene for termination technology in plants.

The genes related to HR repair mechanism have significant roles in cell division in flowers that are the sites occurred recombination during fertilization in plants. XRCC3 functions in the DSB repair pathway in animals. The damaged DNA sites are repaired by recombinational repair with the damaged termini being converted to 3'-OH or 5'-P residue by RAD51 recombinase. In the second phase of the reaction, the gap is filled by DNA ligase. The function interacting the key enzymes such as RAD51, XRCC3 or XRCC4 greatly enhances efficiency in DSB repair and DNA recombination.

On the other side, there are progressing the research about their functions and the genes related in the development and differentiation of pollen, embryo, and seeds. It would be able to apply the basic mechanisms to prevent the unwanted transfer of transgenes from transgenic plants to wild crops. These findings would be helpful to develop a new termination technology not to set seeds by using *XRCC3* gene in transgenic crops.

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