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Original scientific article

NBS-LRR-CONTAINING CLASS OF SALICYLIC ACID-INDUCED GENE TRANSCRIPT IN RYE

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NBS-LRR-type disease resistance gene-like cDNA, induced by salicylic acid (SA) was cloned from rye *Secale cereale* L. (2*n* = 14 RR) var. Petkus, which has rust resistance genes such as *Lr26*, *Sr31* and *Rr9*. We designed primers based on the NBS region and performed PCR using Petkus genomic DNA as a template. Next, we TA-cloned a 532-bp DNA fragment containing five homologous amino acid sequences in the NBS region. The SA-treated rye showed strong expression of a transcript of approximately 3.5 knt in the Northern blots probed with the NBS fragment; however, no transcripts were observed with the untreated rye. We constructed a cDNA library of rye var. Petkus treated with SA, and then screened the cDNA library using the TA-cloned NBS fragments as a probe. The entire nucleotide sequence of a full length of rye NBS-LRR-containing class cDNA 3,446 bp was determined.

Keywords: NBS-LRR, rye, salicylic acid, cDNA, gene cloning, gene structure

INTRODUCTION

Damage by outbreaks of leaf rust (*Puccinia recondita* Roberge ex Desm.) in wheat is a global problem. Rye, *Secale cereale* L. (2*n* = 14 RR), close relative of wheat, contains rust resistance genes such as *Lr26*, *Sr31* and *Rr9* on chromosome 1RS (CRESPO-HERRERA *et al*., 2017). Our aim is to investigate the genes that are the source of this resistance, to enable improving wheat. Plant diseases are determined by the gene-for-gene complementarity theory between the pathogen and its host plant (FLOR, 1956). These resistance gene products recognise

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the pathogenic gene products (effectors) as receptors, and it is thought to act trigger intracellular signal transductions (DEYOUNG and INNES, 2006; MCHALE *et al*., 2006; MARONE *et al*., 2013; MAGO *et al*., 2015). This in turn leads to a resistance response, including the induction of cell death and production of anti-bacterial substances (FU and DONG, 2013).

Plant resistance genes based on gene-for-gene interaction have common features such as Nucleotide binding sites (NBS) and a leucine-rich repeat (LRR) structure, which is believed to recognize virulent gene products (elicitors) and functions in a signaling cascade that induces resistance responses (DEYOUNG and INNES, 2006; MCHALE *et al*., 2006; MARONE *et al*., 2013). Plant's self-protection mechanism known as Systemic Acquired Resistance (SAR) restricts infection through the process of proactive death of cells invaded by the pathogen (hypersensitive cell death) (FU and DONG, 2013). Signals are then transduced to the entire plant and finally production of anti-infection substances ensures the remaining healthy tissue is not invaded by the pathogen. These substances then stimulate the production of proteins that inhibit proliferation and movement of the pathogen. Salicylic acid (SA) is known as a signal transduction in the self-defense mechanism (VLOT *et al*., 2009). In spite of studying several useful resistance genes of rye (MAGO *et al*., 2002), a few genes, such as *Pm8*, *Pm17* have been isolated so far (HURNI *et al.,* 2013; SINGH *et al.,* 2018). The huge genome of rye, 1Cx = 7,917 Mb (BARTOS *et al*., 2008; BAUER *et al*., 2017), have been hindered the cloning of resistance genes.

In the present study, NBS-LRR-type disease resistance gene-like cDNA, induced by salicylic acid (SA) was cloned from the rye variety Petkus. We designed primers based on the NBS region and performed PCR using Petkus genomic DNA as a template. Next, we TA-cloned a 532-bp DNA fragment containing five homologous amino acid sequences in the NBS region. We constructed a cDNA library from the SA-treated Petkus, and then screened the cDNA library using PCR-amplified NBS fragment as a probe. The entire structure of the cDNA clone including NBS-LRR region was determined.

MATERIALS AND METHODS

PCR assay

PCR was performed on Petkus genomic DNA as a template, using primers designed on the consensus NBS region of the plant's NBS-LRR-type gene (Kn2-1 [5'-TGATACTGGATGATGTCTGG, T_m 58.4℃] and Hyd-2 [5'-GTGCTTCTTATGAACCCTTC, T_m 57.8℃]) (DE MAJNIK *et al*., 2013). Using 20 ng of rye genomic DNA as a template, 50 μL of a reaction solution containing 200 nmol/L primer (33 ng), 100 μmol/L dNTPs, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.8), 1.5 mmol/L $MgCl_2$, and 1 U TaKaRa LA Taq (Takara Bio Inc., Kyoto, Japan) was prepared. Using the Thermal Cycler PCR 700 (ASTEC, Fukuoka, Japan), the reaction solution was subjected to 45 cycles of : denaturation at 94°C for 30-s, annealing at 53°C for 30-s, and primer extension at 72°C for 1 min. The first denaturation at 94°C and the last extension at 72°C were set for 5 min. After PCR amplification, 10 μL of the sample was subjected to 1.5% agarose gel electrophoresis at 100 V for 1 h 30 min. The PCR-amplified fragment was cloned with pMOS Blue T vector (GE Healthcare, Buckinghamshire, United Kingdom). Two microliters of a PCR sample solution was gentlymixed with a ligation solution containing 1 μL of 10× ligation solution, 0.5 μL of dithiothreitol (final concentration, 5 mmol/L), 0.5 μL of ATP (final concentration, 0.5 mmol/L), 1 μL of 50 ng/μl pMOSBlue, 4.5 μL of sterile

distilled water, and 0.5 μL (2∼3 units) of T4 DNA ligase. Ligation was carried out at 16°C overnight. One microliter of the above ligation reaction solution was gently mixed with 20 μL of *E. coli* MOSBlue (GE Healthcare, Buckinghamshire, United Kingdom) and incubated for 20 min in an ice bath. The reaction mixture was then pulse treated at 42°C for 90 s, followed by incubation in an ice bath for 2 min. To the reaction mixture, 80 μL of SOC liquid medium prewarmed to 37°C were added and incubated at 37 °C for 30 min. Fifty microliters of the sample medium were spread over an X gal/isopropyl b-D-1-thiogalactopyranoside plate containing 50 μg/mL ampicillin, and the plate was incubated at 37°C for 14 h. Recombinant colonies were picked up using a bacterial colony picker to inoculate 50 mL of LB-amp liquid medium. Plasmid DNA was isolated by alkaline lysis method (BIRNBOIM and DOLY, 1979) and the nucleotide sequence of the inserted DNA was determined by the cycle sequence method.

Northern blot analysis

Salicylic acid (50 mL) was sprayed on one-month-old *Secale cereale* var. Petkus seedlings. Total RNA was extracted from the SA-treated and non-treated plants using the Acid Phenol Guanidium Chloroform method (CHOMCZYNSKI and SACCHI, 2006). RNA (20 μg) from both SA-treated and -untreated Petkus rye leaves, were subjected to 1.5% denatured formaldehyde/agarose gel electrophoresis at 100 V for 1 h 30 min and transferred onto a Biodyne A nylon membrane using a vacuum blotting apparatus. After vacuum transfer, the nylon membrane was dried at 80°C for 10 min, and DNA was crosslinked to the membrane by UV irradiation at 125 mJ. A DNA-linked nylon membrane was incubated in a prehybridization solution (2% blocking reagent; Roche Diagnostics, Basel, Switzerland), 5× SSC, 0.1% N-lauroylsarcosine, and 0.02% sodium dodecyl sulfate (SDS)) for 4 h. PCR products isolated as above were labeled with Dig-11-dUTP (Roche Diagnostics, Basel, Switzerland) by PCR condition described in the PCR section. Next, hybridization was performed at 68°C for 14 h with 50 ng of a labeled 532 bp-DNA fragment including NBS region as a probe in 10 mL of the hybridization solution. The membrane was washed at room temperature in 2× SSC and 0.1% SDS twice for 5 min, and then washed twice at 68℃ for 15 min in 0.1× SSC and 0.1% SDS. The Northern blots on a filter hybridized with DIG-labeled NBS probe, were detected by enzyme immunoassay. After hybridization, the membrane was washed at room temperature twice each with 2× SSC / 0.1% SDS for 5 min and 0.1× SSC / 0.1% SDS for 15 min. The membrane was further washed with washing buffer (0.3% (w/v) Tween 20 / buffer 1, buffer 1:0.1 mol/L maleic acid, 0.15 mol/L NaCl, pH 7.5) for 3 min, blocked for 30 min with buffer 2 (1% (w/v) blocking reagent / buffer 1), and incubated with 1:1000 anti-digoxigenin AP Fab fragments (750 units/mL) in buffer 2 for 30 min. The membrane was washed twice for 30 min with washing buffer and incubated for 5 min in buffer 3 (0.1 mol/L Tris-HCl (pH 9.5), 0.1 mol/L NaCl, and 0.05 mol/L $MgCl_2$). After addition of 2 mL of chloro-5-substituted adamantyl-1,2-dioxetane phosphate (CSPD) solution (10 mg/mL CSPD:buffer 3 = 1:100), the membrane was transferred into a hybridization bag and incubated at 37°C for 10 min. The membrane was then exposed to an X-ray film for 1 h to detect the hybridization of a labeled probe.

Preparing the cDNA library

Poly(A)-RNA was fractionated from total RNA, using the Poly A Tract mRNA Isolation

System (Promega, Madison, WI). The cDNA library was prepared using the ZAP Express cDNA Synthesis Kit (Stratagene, La Jolla, CA). Single-strand DNA was synthesized using T primers with *Xho*I-linked poly(T) primers. Double-stranded DNA was then synthesized. After smoothing both ends, *Eco*RI was added, and *Xho*I digestion was performed. cDNA was synthesized with the addition of the *Eco*RI site at the 5'-terminal and the *Xho*I site at the 3'-terminal. The synthesized cDNA was ligated with λZAP Express Vector (Stratagene), then packaged in vitro into phages with Gigapack Gold packaging extract (Stratagene). An *E. coli* XLI-Blue MRF host was co-infected with λZAP Express phage and ExAssist helper phage. Single-stranded DNA was synthesized from the λDNA, then the cDNA library was prepared by infecting the packaged phagemid with *E. coli* XLOLR. The bacteria were plated on LB plates. Plaque hybridization on the plaques replicated on these plates was performed using the 532 bp-NBS fragments as a probe.

Determining the structure of the cDNA

Phagemid DNA was then synthesized from the resulting two positive phages and deletion clones were prepared. *Sma*I and *Sac*I were used to, respectively, digest the insert DNA side of the plasmid and the sequence primer side, and the *Sma*I blunt end was further digested step-by-step in the direction of the 3′ to 5′ end using exonuclease III to construct nested deletion clones having an approximately 300-bp difference between them. Deletion clones were undergone by cycling sequencing reactions using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, San Jose, CA). Nucleotide sequences of these deletion clones were decoded by using Thermo Fisher ABI Prism Applied Biosystems 3100 Genetic Analyzer. The entire nucleotide sequence of the cDNA was then determined by assembling overlapped read sequences. Nucleotide sequences were compared with sequences in the non-redundant GenBank+EMBL+DDBJ databases with BLASTN homology search software (ALTSCHL *et al*., 1997). A phylogenetic tree was constructed with Phylogenetic analysis pipeline by ETE3 (HUERTA-CEPAS *et al*., 2016).

RESULTS

NBS-LRR-type fragments: cloning and expression analysis

PCR was performed by 20-mer primer set for the NBS region on the genomic DNA of rye variety Petkus as template. The amplified DNA fragment was 532 bp contained three amino acid homologous sequences for domains 1, 2, and 3, in common with the NBS region, for example, the flax rust resistance gene *L6* (ELLIS *et al*., 1999), the *Aegilops tauschii* Coss. nematode resistance gene *Cre3* (MAJNIK *et al*., 2004), the *Arabidopsis* bacterial resistance gene *RPS2* (MINDRINOS *et al*., 1994), and the tobacco resistance gene *N* (LAWRENCE *et al*., 1995), etc. (Figure 1). It showed an 88.5% homology with the nucleotide sequence for the *Aegilops tauschii Cre3* gene.

We performed northern hybridization on the total RNA, which was extracted by the AGPC method, 48 h after spraying young Petkus plants with 50 mM of SA solution. Northern blots using a 532-bp NBS fragment as a probe showed strong transcription of 3.5 knt as well as to 0.6-4.8 knt RNAs in the SA-treated plant; however, no hybridization was observed with the untreated plant (Figure 2). It was inferred that SA treatment strongly induced transcription of several NBS-LRR-type genes.

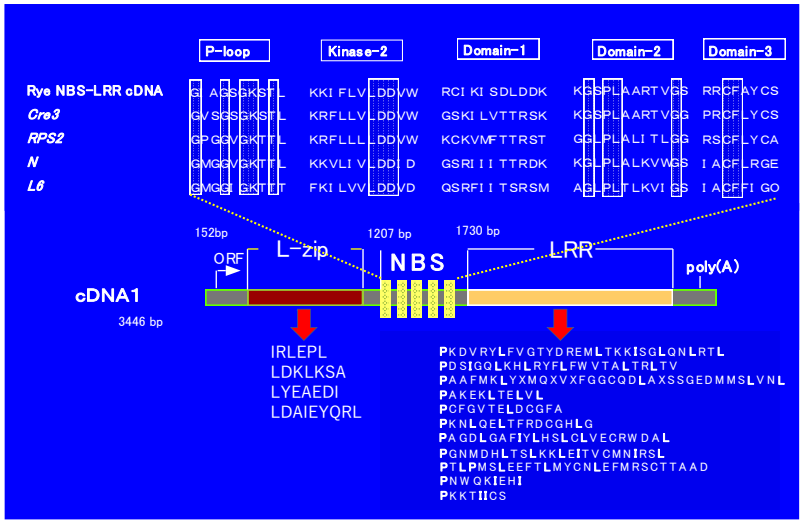

Figure 1. Full structure of rye NBS-LRR containing class cDNA. (A) Conserved NBS-LRR domains. (B) Leucine zipper structure. (C) Leucine-rich repeats. The entire nucleotide sequence of cDNA was 3,446 bp, which contained the 532 bp region coinciding with the NBS probe starting at a point 1,207 bp from the 5' end (GenBank accession number MN495622). Behind the NBS region, there existed 222 aa of leucine-rich repeats. The NBS-LRR-type cDNA contained putative single open reading frame of 984 aa. The upstream of the NBS region included a leucine zipper structure, in which five leucine appears repeatedly after every seven amino acid residues, as following [5-IRLEPLLDKLKSALYEAEDILDAIEYQRL-3].

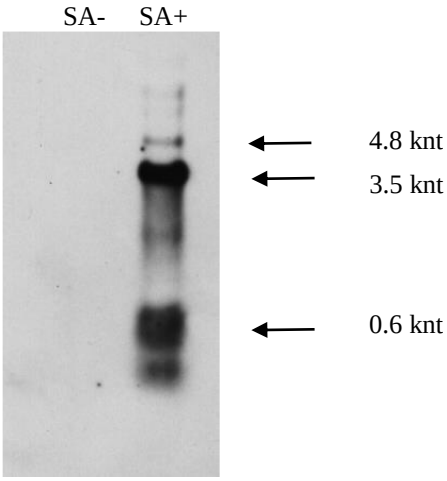

Figure 2. Northern blot analysis of rye NBS-LRR probe against SA-treated leaves. The 532-bp NBS fragment as a probe showed strong transcription of 3.5 knt as well as to 0.6-4.8 knt RNAs in the SA-treated plant; however, no transcription was observed with the untreated plant. SA+: Total RNA extracted from leaves treated with 50 mM SA for 48 h, SA-: Total RNA extracted from control leaves.

Cloning and structural analysis of full-length cDNA with NBS-LRR domain from SA-treated rye

cDNA was synthesized by reverse transcriptase from poly(A) mRNA strands fraction from the total RNA of SA-treated rye. The cDNA was ligated to λZAP Express vector, packaged in vitro to phage particles. The phages were then infected to XL1-Blue MRF' strain, to prepare a 1.4×10^6 pfu cDNA library. Using a 532-bp NBS fragment as a probe, nine positive phages were selected by colony hybridization to approximately 250,000 plaques of the SA-induced cDNA library, and further narrowed these down to two clones through secondary screening.

The nucleotide sequences of the two positive clones screened from the cDNA library of the SA-treated plants were identical. The entire nucleotide sequence of cDNA was 3,446 bp, which contained the 532 bp region coinciding with the NBS probe starting at a point 1,207 bp from the 5' end (Figure 1, GenBank accession number MN495622). Behind the NBS region, there existed 222 aa of leucine-rich repeats. The NBS-LRR-type cDNA contained putative single open reading frame of 984 aa. The upstream of the NBS region included a leucine zipper structure, in which five leucines appears repeatedly after every seven amino acid residues, as following [5-lRLEPLLDKLKSALYEAEDILDAIEYQRL-3]. This is an important pattern for hydrophobic interactions. In addition, there were two hydrophobic regions on the cDNA, upstream from the NBS region, including 10 amino acid residues ([5-AAVGMGVSAV-3] and [5-YEAEDILDA-3]) not found in other NBS-LRR resistance genes.

DISCUSSION

The NBS-LRR-type cDNA was a new sequence, as it showed no sequence homology with GenBank sequences outside the NBS region. There were several homologies with disease resistance-like genes such as *Aegilops tauschii* RPP-13 [F775_13548], RGA-3 [ats_109762416], *Oryza sativa* RGA-2 [LOC9269167], *Zea mays* RGA1 [XM_020551773.2] (Figure 3). However, these were incomplete sequences shorter than the rye NBS-LRR type cDNA, because they lacked 5', or 3' parts, and both ends (Figure 3). There is no homologous sequence of rye, *Secale cereale* L in GenBank. We have successfully isolated the longest full sequence of rye NBS-LRR type cDNA among the isolated or data base sequences. The total length of 3,446 bp was well in accord with the strongest transcript induced by SA (Figure 2). The rye NBS-LRR containing cDNA is thought to be a major transcript by SA treatment. In the phylogenetic tree, rye NBS-LRR type cDNA is most related to RPP-13 class of disease resistance genes of *Aegilops tauschii* [F775_10432, F775_13548] (Figure 3). The rye cDNA was hydrophobic and 54% at the 5'-terminal upstream from the NBS. Homology was especially high in the hydrophobic region and lower downstream of the NBS.

In this study, we isolated a gene that had basic two structures NBS and LRR for resistance genes, from SA-treated rye, a close relative and useful genetic resource of wheat. Generally, the NBS-LRR-type gene is thought to function by binding of the non-pathogenic gene products of the pathogen with plant proteins caused by pathogenic damage, through hydrophobic interactions on the LRR region of these plant proteins (DEYOUNG and INNES, 2006; MCHALE *et al*., 2006; MARONE *et al*., 2013). In addition, it is thought that this activates phosphorylase, leading to a resistance response, whereby intracellular and extracellular signals are initiated (FU and DONG, 2013). The rye NBS-LRR type cDNA sequence would be a key sequence to get rye substantial disease resistance genes.

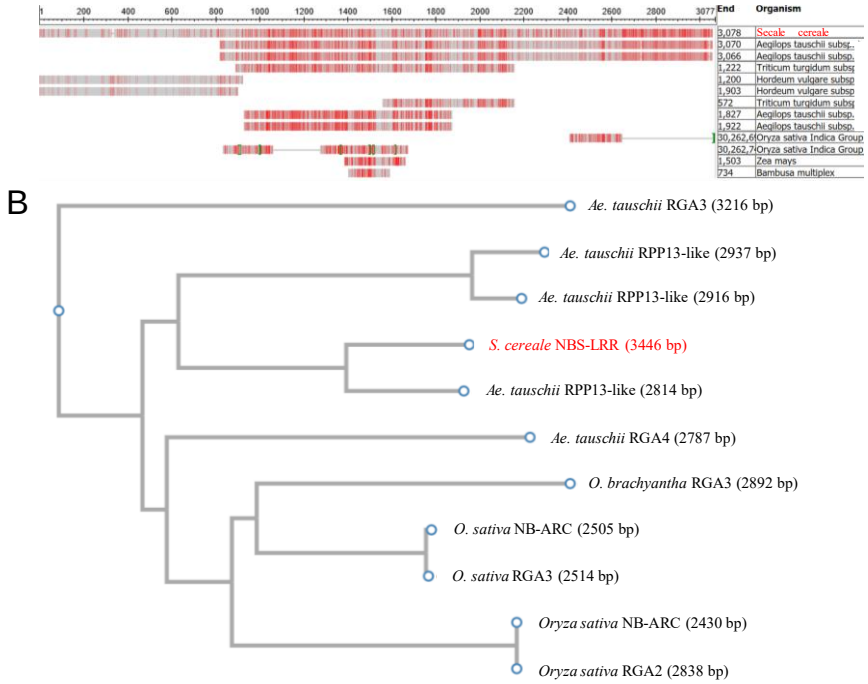

Figure 3. Relationship among homologous genes with rye NBS-LRR containing class cDNA. (A) Structural relationship. The NBS-LRR-type cDNA was a new sequence, as it showed no sequence homology with GenBank sequences outside the NBS region. There were several homologies with disease resistance-like genes such as *Aegilops tauschii* RPP-13 [F775_13548], RGA-3 [ats_109762416], *Oryza sativa* RGA-2 [LOC9269167], *Zea mays* RGA1 [XM_020551773.2], which were however incomplete sequences shorter than the rye NBS- LRR type cDNA, because they lacked 5', or 3' parts, and both ends. (B) Phylogenetic relationship. The rye NBS-LRR type cDNA is most related to RPP-13 class of disease resistance genes of *Aegilops tauschii* [F775_10432, F775_13548].

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NBS-LRR KLASA TRANSKRIPTA GENA INDUKOVANIH SALICILNOM KISELINOM KOD RAŽI

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Izvod

NBS-LRR tip otpornosti na bolesti indukovan salicilnom kiselinom (SA) klonirana je iz raži *Secale cereale* L. (2n = 14 RR) var. Petkus, koja ima gene za otpornost na rđu kao što su Lr26, Sr31 i Rr9. Dizajnirali smo prajmere zasnovane na NBS regionu i uradili PCR koristeći Petkus genomsku DNK kao obrazac. Zatim smo TA klonirali 532 bp fragment DNK koji sadrži pet homolognih aminokiselinskih sekvenci u NBS regionu. Raž tretirana SA pokazala je snažnu ekspresiju transkripta od približno 3,5 knt u *Northern blots*-u sa NBS fragmentom; međutim, nisu primećeni transkripti kod netretirane raži. Konstruisali smo cDNA biblioteku raži var. Petkus tertian SA, a zatim je urađen skrining biblioteke cDNA koristeći TA-klonirane NBS fragmente kao probu. Utvrđena je cela nukleotidna sekvenca pune dužine ražene NBS-LRR klase koja sadrži cDNA 3,446 bp.

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