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. (2n = 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. (2n = 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 INNESS, 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 INNESS, 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 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, Tm 58.4°C] and Hyd-2 [5’-GTGCTTCTCTATGAACCCTTC, Tm 57.8°C]) (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 MgCl2, 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 gently mixed 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
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Preparation of 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 XhoI-linked poly(T) primers. Double-stranded DNA was then synthesized. After smoothing both ends, EcoRI was added, and XhoI digestion was performed. cDNA was synthesized with the addition of the EcoRI site at the 5’-terminal and the XhoI 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 (ALTSCHUL 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.
NBS motifs of the NBS-LRR containing class of R genes
Cre3
RPS2

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 region included a leucine zipper structure, in which five leucine repeats (IRLEPLLKLK) appeared repeatedly after every seven amino acid residues, as following [5IRLEPLLKLKSALEAEDILDAIEYQR-3].

Figure 1. Full structure of rye NBS-LRR containing class cDNA. (A) Conserved NBS-LRR domains. (B) Leucine zipper structure. (C) Leucine-rich repeats.

SA-   SA+

4.8 knt

3.5 knt

0.6 knt

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-LRLEPLDLKLKSALYEAEDILDAIEYQRL-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-AAVGMGVS-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 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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