Isolation and characterization of fusarium wilt resistance gene analogs in radish

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Abstract

The resistance gene analog (RGA)-based marker strategy is an effective supplement for current marker reservoir of radish disease-resistance breeding. In this study, we identified RGAs based on the conserved nucleotide-binding site (NBS) and S-receptor-like kinase (SRLK) domains. A total of 68 NBS-RGAs and 46 SRLK-RGAs were isolated from two FW-resistant radish inbred lines, B2 and YR31, and one susceptible line, YR15. A BLASTx search revealed that the NBS-RGAs contained six conserved motifs (i.e., P loop, RNBS-A, Kinase-2, RNBS-B, RNBS-C, and GLPL) and the SRLK-RGAs, contained two conserved motifs (i.e., G-type lectin and PAN-AP). A phylogenetic analysis indicated that the NBS-RGAs could be separated into two classes (i.e., toll/interleukin receptor and coiled-coil types), with six subgroups, and the SRLK-RGAs were divided into three subgroups. Moreover, we designed RGA-specific markers from data-mining approach in radish databases. Based on marker analysis, 24 radish inbred lines were clustered into five main groups with a similarity index of 0.44 and showing genetic diversity with resistance variation in those radish inbred lines. The development of RGA-specific primers would be valuable for marker-assisted selection during the breeding of disease-resistant radish cultivars.

Keywords Resistance gene analogs (RGA) · Fusarium wilt · Nucleotide-binding site (NBS) · S-receptor-like kinase (SRLK)

Introduction

Fusarium wilt (FW) widely recognized as one of the most destructive fungal disease influencing radish (*Raphanus sativus* L.; $2n=18$) yield and quality, which is caused by *Fusarium oxysporum*. Currently used chemical treatments to control this disease are harmful to the environment. During the last two decades, several quantitative trait loci (QTLs) and genes conferring FW resistance have been mapped and isolated in diverse plants, including *Arabidopsis thaliana*

(Cole and Diener 2013; Diener and Ausubel 2005; Shen and Diener 2013), Chinese cabbage (Shimizu et al. 2014), cabbage (Shimizu et al. 2014; Lv et al. 2014), tomato (Catanzariti et al. 2015; Gonzalez-Cendales et al. 2016; Simons et al. 1998; Wei et al. 2014) as well as radish (Yu et al. 2013). However, until now, the underlying genetic basis of the disease-resistance mechanisms in radish has not been fully characterized, especially the disease-resistance genes (*R* genes). Most *R* genes have similar structures and encode conserved motifs, including the nucleotide-binding site (NBS), Toll/interleukin-1 receptor (TIR), coiled-coil (CC), and protein receptor-like kinase (RLK) (Ellis et al. 2000; Jones and Jones 1997; McHale et al. 2006). The conserved regions of different *R* genes may be useful for developing degenerate marker-based strategies to isolate and map *R* genes or their analogs.

Molecular technologies in modern breeding have accelerated the breeding of disease-resistant plants. For example, FW-resistance genes have been cloned in tomato, including *I-2*, which encodes a CC–NBS–leucine-rich repeat (LRR) protein (Simons et al. 1998; Wei et al. 2014), *I-3*,

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which encodes an S-receptor-like kinase (SRLK) (Catanzariti et al. 2015), and *I-7* (Gonzalez-Cendales et al. 2016), which encodes an Leucine-rich repeat-receptor-like protein LRR–RLP (i.e., another *R* gene class). In addition, *A. thaliana* FW-resistance genes have been identified such as *RFO1*, which encodes a cell-wall-associated receptor-like kinase (Diener and Ausubel 2005), *RFO2*, which encodes an LRR–RLP similar to *I-7* (Shen and Diener 2013), and *RFO3*, which encodes an SRLK similar to *I-3* (Cole and Diener 2013). In *Brassica oleracea*, the candidate gene *FocBol* reportedly encodes an NBS–LRR (Lv et al. 2014; Shimizu et al. 2014). In most plant–pathogen systems, *R* genes conferring resistance to different pathogen isolates belong to just one or two classes. The multiple classes of FW-resistance genes imply that the *F. oxysporum* infection process (inside and outside plant cells) is complex. They also provide diverse opportunities to study plant disease-resistance mechanisms (Gonzalez-Cendales et al. 2016). Therefore, the isolation and characterization of radish *R* genes are essential for the breeding of disease-resistant radish varieties.

There are at least three different classes of genes are found which provide resistance against *F. oxysporum*, this implies that the complex nature of associated FW disease-resistance mechanism. Plant RGA markers were designed according to the conserved sequence of known RGAs, which in a resistance gene are conserved in different species. The property of RGA sequences is actually not the resistance genes, but could help breeders to identify resistance genes over species (Tomar et al. 2010). In this study, we selected the two main classes of *R* genes responsible for FW resistance (i.e., NBS–LRR and S-domain RLK classes), which were simultaneously identified in tomato and *A. thaliana*. A polymerase chain reaction with degenerate primers was used to target the sequences encoding the conserved motifs of the NBS and SRLK domains following a comparison of known *R* genes, including those from tomato, *A. thaliana*, and *Brassica rapa*. We isolated 68-resistance gene analogs of the NBS domain and 46 RGAs of the SRLK class in radish. Furthermore, we developed polymorphism markers and examined in 24 radish inbred germplasms exhibiting varying FW-resistance levels. These RGA-specific markers developed may be useful for marker-assisted selection during the breeding of disease-resistant radish cultivars.

Materials and methods

Plant materials and DNA extraction

The FW-resistant inbred lines YR31 and B2 and FW-susceptible inbred line YR15 analyzed in this study were grown in plastic pots in a greenhouse at Chungnam National University on September 2016. Genomic DNA was extracted

from seedling leaf samples using the Exgene™ Plant SV kit (GeneAll, Seoul, Korea) for a subsequent isolation of RGAs. The genetic variability in 24 radish inbred lines was investigated using RGA-specific markers. All 24 radish lines were inoculated using a root-cut dipping method, as described by Yu et al. (2013). The disease index scores are provided in Table 1.

PCR amplification, cloning, and sequencing

Degenerate primers were designed based on the conserved region of the NBS–LRR *R* gene between the sequences encoding the P loop and GLPL described in the previous studies (Deng et al. 2000; He et al. 2004; Sharma et al. 2009; Wang et al. 2014). Two forward primers were designed: 5'-GGYATGGGNGGYMTHGGNAARAC-3' (Wang et al. 2014) and 5'-TGSSRGGHWHYRGGBAAACTAC-3' (Wan

Table 1 The 24 radish genotype used in this study

No.	Source	Line name	Grade of disease symptoms ^a	Resistant type ^b
1	Syngenta Co.	B2	2.5 ± 1.8	MR
2		835	6.4 ± 1.7	S
3	Neoseed Co.	YR1	1.2 ± 0.7	MR
4		YR2	0.7 ± 0.5	R
5		YR3	3.1 ± 1.0	S
6		YR4	0.7 ± 0.8	R
7		YR5	0.8 ± 0.4	R
8		YR6	1.0 ± 0.9	R
9		YR7	1.9 ± 1.3	MR
10		YR8	1.8 ± 0.9	MR
11		YR9	3.0 ± 1.3	MR
12		YR10	1.5 ± 0.9	MR
13		YR11	1.2 ± 1.6	MR
14		YR12	2.4 ± 1.7	MR
15		YR13	4.5 ± 0.7	S
16		YR14	5.0 ± 0.0	S
17		YR15	3.8 ± 0.4	S
18		YR16	4.7 ± 0.6	S
19		YR17	4.6 ± 1.2	S
20		YR18	5.0 ± 0.0	S
21		YR22	1.8 ± 1.1	MR
22		YR29	1.0 ± 0.9	R
23		YR31	0.6 ± 0.5	R
24		YR35	1.2 ± 1.0	MR

R, MR, and S indicated resistance, mild resistance, and susceptibility to FW, respectively

^aAverage of disease symptoms for each line containing ten plants (bold number) ± standard deviation

^bResistance: 0 < Average ≤ 1, mild resistance: 1 < Average ≤ 3.0, susceptibility: Average > 3.0

et al. 2012) as well as one reverse primer: 5'-GAGGGCTAAAGGAAGGCC-3' (Deng et al. 2000). In addition, SRLK *R* gene degenerate primers were designed according to the conserved G-lectin and PAN-AP sequences (i.e., forward primers: 5'-GTYTGGWYTGCTAACMGKAAC-3' and 5'-CAAGAACAGTKWYKBAGACA-3'). The details of the degenerate primers are provided in Table 2. The PCR products corresponding to the expected size were purified using the AccuPrep® Gel Purification Kit (Bioneer, Korea) and cloned into the T-Blunt according to the manufacturer recommended instructions (Biofact, Korea). The cloned PCR fragments were sequenced at SolGent Company, Korea.

Sequence alignment, BLAST search, and phylogenetic analysis

A BLAST search was used to detect sequences homologous to the deduced NBS-LRR and SRLK amino acid sequences in the National Center for Biotechnology Information GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST>). Sequences were aligned using the CLC Main Workbench 5.0 (CLC bio, Aarhus, Denmark). Phylogenetic trees were constructed according to the maximum-likelihood principle which performed for the values of the free parameters in the model assumed that results in the highest likelihood of the observed alignment (Felsenstein 1981). The starting tree was generated based on the neighbor-joining method, using the Jukes-Cantor substitution model with 1000 bootstrap replicates.

Data mining of the radish database for *R* genes and development of RGA markers

The RGA-specific markers were designed based on conserved sequences among seven groups of NBS-RGAs and SRLK-RGAs using the Primer3 online tool (<http://bioinfo.ut.ee/primer3-0.4.0/>). The sequences of 12 known FW *R* genes from (*A. thaliana*, (*B. rapa*, and *B. oleracea*, including four *R* gene types, were used in a BLAST search of three radish databases to identify homologous sequences (i.e., <http://bioinfo.bti.cornell.edu/cgi-bin/radish/index.cgi>, <http://radish.kazusa.or.jp/>, <http://www.radish-genome.org/>). The detected radish homologs that were likely to have *R*

gene-like functions were used to develop RGA-specific markers (Supplementary Table 1). In addition, from our previous report, the nine markers in FW major QTLs region selected to involvement (Yu et al. 2013). All primer pairs were validated using 24 radish inbred lines. The expected bands were scored for constructing a dendrogram by the unweighted pair-group mean algorithm (UPGMA) clustering with NTSYS-pc 2.11 software.

Results

Amplification of RGAs

We used two combinations of degenerate primers designed based on six previously described conserved regions in NBS-encoding *R* genes (i.e., encoding the P loop, RNBS-A, Kinase-2, RNBS-B, RNBS-C, and GLPL motifs). Using T-Blunt vector cloning, 30 positive clones for each inbred line were randomly selected and sequenced. The BLAST results confirmed that the sequences corresponded to parts of the radish NBS-LRR and SRLK domains, and the BLASTx analysis revealed that 19, 26, and 23 of the NBS-RGAs from B2, YR15, and YR31, respectively, were highly similar to known *R* genes and RGAs from other species (Supplementary Table 2). The sequences of the remaining clones did not match any *R* genes. We also used one degenerate primer pair which was designed according to conserved G-lectin and PAN-AP sequences of the SRLK-encoding *R* gene. After cloning, 30 positive clones were randomly selected for sequencing. The BLASTx results indicated that 18, 20, and 8 SRLK-RGAs from B2, YR15, and YR31, respectively, were highly similar to known *R* genes and RGAs from other species (Supplementary Table 2).

Multiple sequence alignment and phylogenetic analysis of NBS-encoding RGAs

A sequence alignment involving the 68 NBS-RGAs and known FW *R* genes (i.e., *Fcobo1* from *B. oleracea* and Bra012688 from *B. rapa*) revealed six conserved sequences, encoding the P loop, RNBS-A-TIR or non-TIR, Kinase-2, RNBS-B, RNBS-C, and GLPL (Fig. 1).

Table 2 Information of degenerate primers

Degenerate primer	Sequence	Size (bp)	Conserved domain	References
Ploop-F1	TGSSRGGHWYRGGBAAACTAC	22	(A/G/P/R)G(T/I/M/S/L)GKTT	Wan et al. (2012)
Ploop-F2	GGYATGGGNGGYMTHGGNAARAC	23	GMGGLGKT	Wang et al. (2014)
GLPL-R1	GAGGGCTAAAGGAAGGCC	18	GLPLAL	Deng et al. (2000)
G-lectin-F	GTYTGGWYTGCTAACMGKAAC	21	VW(F/I/S)AN(S/R)NNP	
Pan-ap-R	CAAGAACAGTKWYKBAGACA	20	RAMCL

Fig. 1 Amino acid sequence alignment of 29 representative radish RGAs (RsRGAs) and two NBS classes of *R* genes in *B. rapa* and *B. oleracea*. **a** NBS-TIR amino acid sequence alignment. **b** NBS-non-TIR amino acid sequence alignment

In addition, we observed that the Kinase-2 motif amino acid sequence contained a conserved aspartic acid residue in the TIR-type *R* genes and a conserved tryptophan residue in the non-TIR-type *R* genes. Based on these typical characteristics, all NBS-RGAs described herein were divided into non-TIR (i.e., CC types) and TIR subclasses. The NBS_YR15-5, NBS_YR15-6, NBS_YR15-10, NBS_YR15-11, and NBS_YR15-26 RGAs belonged to the non-TIR subclass, while the remaining 63 RGAs were included in the TIR subclass. The constructed phylogenetic tree was

consistent with the results of the amino acid sequences analysis. The two major branches of the phylogenetic tree were comprised of the non-TIR–NBS–LRR and TIR–NBS–LRR genes (Fig. 2). Within the TIR–NBS–LRR subfamily, the NBS-RGAs could be separated into six groups (i.e., Groups 1–6). Each group consisted of different numbers of RGAs, and was associated with a unique conserved sequence. The *Fcobo1* and *Bra012688* genes from *B. oleracea* and *B. rapa*, respectively, were related to the Group 6 members. Group 7 consisted of only the

Fig. 2 Phylogenetic tree of NBS domain (P loop to GLPL) RGAs in three different radish, B2, YR15, and YR31 and two FW *R* genes in *B. oleracea* and *B. rapa*. The construction method is neighbor-joining

with 1000 times replicates. Numbers above branches are bootstrap support values (Bold lines: shown where > 50)

non-TIR–NBS–LRR subfamily which from the FW-susceptible line YR15 (Fig. 2).

Multiple sequence alignment and phylogenetic analysis of SRLK-encoding RGAs

Forty-six SRLK-encoding RGAs from lines B2, YR15, and YR31 were aligned with the SRLK genes *RFO3* from (*A. thaliana*) and *Br027195* from (*B. rapa*) (Fig. 3). We observed that all of the SRLK-RGAs contained conserved sequences corresponding to the G-lectin and PAN-AP motifs. A phylogenetic tree (Fig. 4) was constructed based on the amino acid sequences encoded by these RGAs as well as *RFO3* and *Br027195*. According to the amino acid sequence similarities, the radish SRLK-RGAs were classified into three groups. Groups 1 and 2 comprised of SRLK-RGAs from the inbred lines B2 and YR15. In contrast, the entire Group 3 SRLK-RGAs were from the inbred line YR31 that has a unique insertion of eight-amino acid which absent in the Group 1 and 2 members. In addition, *RFO3* and *Br027195* formed an independent branch not associated with the three groups.

Development of RGA-specific markers and analysis of genetic variability

The known FW *R* genes in *Brassica* species were used to perform the BLAST search in the three radish sequence databases (Supplementary Table 1). Sixteen pairs of RGA-specific primers were designed based on the seven identified groups of RGA sequences and BLAST search results (Supplementary Tables 2, 3). PCR analysis of 24 radish inbred lines with varying levels of FW resistance (Table 1) revealed that three out of the 16 RGA-specific markers generated polymorphic bands. The RsSRLK-Indel marker was designed based on a 24-bp insertion found only in the YR31 sequence. The RsFor1 marker targeted the *A. thaliana* FW *R* gene *ATFor1*, whereas the RsGRP3 marker was developed based on the Group 3 NBS RGA sequences. The PCR amplification product profiles for the 24 radish inbred lines are presented in Supplemental Fig. 1.

Genetic diversity analysis

The RGA-specific markers and 9 SSR makers that selected from FW major QTL regions in a previous study (Yu et al.

Fig. 3 Amino acid sequence alignment of 46 radish RGAs (RsRGAs) and two SRLK classes in (*A. thaliana*) and (*B. rapa*)

Fig. 4 Phylogenetic tree of SRLK domain (G-lectin to PAN_AP) RGAs in three different radish, B2, YR15, and YR31. The construction method is neighbor-joining with 1000 times replicates. Numbers above branches are bootstrap support values (Bold lines: shown where > 50)

2013) were used for genetic diversity analysis in the 24 different FW-resistance levels of radish. Cluster analysis based on the UPGMA, in which the genetic similarity coefficient ranged from 0.44 to 0.95 (Fig. 5). These 24 radish inbred lines were clustered into five main clusters at a similarity index value of 0.44. Cluster I consisted of 7 radish inbred lines, one is 835 with susceptible and six inbred lined with mild resistance and high resistance. Cluster II was divided into two subgroups at a similarity index value

of 0.77, Subgroup I contained 5 inbred lines, in which two showed mild resistance and three were highly resistance. Subgroup II included two inbred lines, YR13 and YR14 both showed susceptibility to FW. Cluster III consisted of one susceptible line YR17 and one resistant line YR16. Cluster IV composed of three inbred lines: two susceptible (YR15 and YR16) and one resistant (YR8). Cluster V consisted of four inbred lines which were considered as mildly resistant and only one susceptible YR18.

Fig. 5 Dendrogram of 24 radish inbred lines based on cluster analysis of polymorphic RGA-based markers and SSR markers

Discussion

Resistance gene analogs have been PCR amplified and cloned from different plant species *via* conserved functional domains (Deng et al. 2000; He et al. 2004; Yaish et al. 2004). In the present study, radish RGA sequences were isolated from genomic DNA using degenerate primers. The FW-resistant inbred lines B2 and YR31 as well as the FW-susceptible line YR15 were used to isolate RGAs. Line B2 was previously used as a parental line for constructing a genetic map and analyzing FW-resistance QTLs (Yu et al. 2013). Line YR31 exhibited the highest level of FW resistance during the screening of disease-resistant resources. A total of 68 and 42 RGAs for the NBS and SRLK sequences were cloned from the three inbred lines. The structures and encoded amino acid sequences were highly conserved among the different cloned *R* genes. In addition, most of the isolated RGAs were highly homologous to disease-resistance-like genes in *B. rapa* and *A. thaliana*. The cloned radish RGAs may provide useful genomic information to disease-resistance identification and accelerate breeding disease-resistant radish cultivars.

Plants are susceptible to diseases caused by fungi, viruses, bacteria, and nematodes. Breeding new disease-resistant cultivars is considered as one of the ideal ways to control plant diseases. Candidate genes have been grouped

into two categories according to resistance and defense functions (Sharma et al. 2009). The candidate genes of one group are activated during the initial recognition of the plant pathogen (i.e., *R* genes). Genes encoding the NBS–LRR proteins belong to this category. The encoded CC and TIR regions influence the recognition of pathogen-specific genes. These two regions differ regarding sequences and the associated signaling pathways (Pan et al. 2000). We cloned these two types of RGAs. However, the CC–NBS was only detected in line YR15, implying that the NBS sequence differed between FW-resistant and FW-susceptible radish inbred lines.

The second group of candidate genes is related to the defense responses that are induced after a pathogen has been detected by the host plant (i.e., *DR* genes) (Dixon and Harrison 1990). Examples include the SRLK-encoding genes. The S-domain of SRLK contains the G-type lectin and PAN-AP motifs, cysteine residues, and the PTDT motif. In most plant species, the SRLK genes function in self-compatibility responses, with various effects on plant growth and development (Dwyer et al. 1994; Tobias et al. 1992). Nonetheless, Ohtake et al. (2000) and Pastuglia et al. (2002) observed that plant defense and signaling activities up-regulated the expression of SRLK-encoding genes in response to pathogen infections. (Ohtake et al. 2000; Pastuglia et al. 2002). It could be due to existence of at least two disease-resistance parallel defense signaling

pathways in YR31 and B2, which preferential utilization by NBS-LRR proteins that are distinguished by their TIR or CC domains (Aarts et al. 1998; Warren et al. 1999). Genes encoding protein kinases constitute a large family and are essential for various plant physiological and biochemical activities in response to hormones and environmental stimuli. The known kinase-encoding plant genes include *I3* of tomato, *RFOR3* of *A. thaliana*, and *RPG1* of barley (Brueggeman et al. 2002; Catanzariti et al. 2015; Cole and Diener 2013). Twelve conserved motifs have been identified in kinases with diverse functions (Hanks and Hunter 1995). In this study, we cloned 46 radish SRLK RGA sequences based on these conserved motifs. These RGAs were divided into three groups, which indicated they were highly diverse. In particular, one group included SRLK-RGAs only from line YR31. Thus, these SRLK-RGAs may represent the *R* genes in line YR31. However, analyses of the known (*A. thaliana*, (*B. rapa*, and tomato SRLK-encoding genes and the predicted amino acid sequences suggest that these genes originated from a common ancestor, with the changes in the encoded amino acid sequences occurring during evolution.

The RGA markers might restrict the polymorphism in the radish genome more than the simple sequence repeat or single-nucleotide polymorphism markers. This may explain why only three RGA-specific markers were validated in this study. However, RGA markers may be useful for characterizing the genetic diversity of several *R* genes in plants, and for applying candidate genes to the mapping of disease-resistance QTLs. Several primer pairs were designed for the RGAs and *R* genes (including unigenes/expressed sequence tags) and subsequently validated using 24 radish inbred lines. The results revealed that there is a low association between the FW-resistance evaluation and cluster analysis. Because of the following reasons: (a) complexity of the disease-resistance mechanism in FW, resistance was controlled by multiple genes; (b) the existence of race-specific genes mediating FW resistance; (c) the primary mapping regions of FW are 10 cM and contained hundreds of genes. It is impossible to evaluate all of these positional candidates for possible disease related polymorphism; (d) the data-mining candidates just on the basis of known or inferred resistance which also limited studies in FW resistance; and (e) pathogens interact with environmental factor (i.e., FW primarily affected by temperature) could miss many relevant genes. The two isolated and characterized RGA categories may be useful for clarifying the complex mechanism underlying FW resistance. In addition, these RGAs may be relevant for parental line selection in a cross to breed new radish cultivars that are highly resistant to FW, which could decrease the need to apply environmentally harmful fungicides during the cultivation of radish.

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Author Contributions XY designed the study, interpreted all data and drafted the manuscript. DHK carried out the RGA cloning; sequencing data analyzed and interpreted all data. SRC interpreted all data and drafted the manuscript. YM and LL carried out primer designing, and carried out the marker development. SHO performed the marker survey and genotyping in 24 radish inbred lines. SSC checked for English grammar and interpreted the research data to modify the manuscript. YPL conceived the study, participated as a director, and helped to draft the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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