Gene xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Molecular characterization of *ZzR*1 resistance gene from *Zingiber zerumbet* with potential for imparting *Pythium aphanidermatum* resistance in ginger

R. Aswati Nair [a,*], George Thomas [b]

[a] School of Biotechnology, National Institute of Technology Calicut (NITC), Calicut, Kerala, India
[b] Spice Genomics Group, Rajiv Gandhi Centre of Biotechnology, Trivandrum, Kerala, India

ARTICLE INFO

Article history:
Accepted 6 December 2012
Available online xxxx

Keywords:
CC-NBS-LRR
Zingiber zerumbet
Ginger
Resistance gene
Pythium spp.
Genome walker

ABSTRACT

Soft rot disease caused by the oomycete *Pythium aphanidermatum* (Edson) Fitzp. is the most economically significant disease of ginger (*Zingiber officinale* Rosc.) in tropical countries. All available ginger cultivars are susceptible to this pathogen. However a wild ginger relative *viz*., *Zingiber zerumbet* L. Smith, was identified as a putative soft rot resistance donor. In the present study, a putative resistance (R) gene designated, *ZzR*1 was isolated and characterized from *Z. zerumbet* using sequence information from *Zingiber* RGCs identified in our earlier experiments. Analysis of the 2280 bp segment revealed a 2157 bp open reading frame (ORF) encoding a putative cytoplasmically localized protein. The deduced *ZzR*1 protein shared high homology with other known R-genes belonging to the CC-NBS-LRR (coiled coil-nucleotide binding site-leucine rich repeat) class and had a calculated molecular weight of 84.61 kDa. Real-time PCR analysis of *ZzR*1 transcription in *Z. zerumbet* following pathogen infection demonstrated activation at 3 hpi thus suggesting an involvement of *ZzR*1 in *Z. zerumbet* defense mechanism. Although many R-genes have been characterized from different taxa, none of them will help in the development of resistant ginger cultivars owing to the phenomenon of "Restricted Taxonomic Functionality" (RTF). Thus *ZzR*1 gene characterized from the resistant wild *Zingiber* accession represents a valuable genomic resource for ginger improvement programs. This first report on R-gene isolation from the *Zingiber* secondary gene pool is pivotal in designing strategies for engineering resistance in ginger, which is otherwise not amenable to conventional improvement programs owing to sexual reproduction barriers.

1. Introduction

Ginger, (*Zingiber officinale* Rosc.), a herbaceous perennial of the family Zingiberaceae is an important export-oriented spice crop with high medicinal value (Afzal et al., 2001). Ginger production is seriously affected by many bacterial and fungal diseases of which soft rot caused by *Pythium aphanidermatum* (Edson) Fitz. is highly destructive (Dake and Edison, 1989). *Pythium* spp. cause highly destructive diseases in various other crops also and are difficult to manage due to enduring spore viability and broad host range that contributes to persistence of *Pythium* spp. in cropping soils (McCarter and Littrell, 1970; Weiland, 2003). Resistance sources have so far not been identified in ginger germplasm against this pathogen (Sarma, 1994). Ginger is completely sterile and is propagated exclusively by vegetative means using rhizome. Complete sterility together with the poor variability for disease resistance hinders the application of any conventional methods for breeding resistance in ginger thereby necessitating the adoption of transgenic technology for crop improvement. Earlier studies have reported *Z. zerumbet*, a wild congener of the cultivated ginger as a potential donor of soft rot resistance for the genetic improvement of ginger (Kavitha and Thomas, 2007).

Shaped by continuously evolutionary battle against microbial invaders, plants have developed a sophisticated immune system to combat the invading pathogens. According to the gene-for-gene model, specific pathogen recognition is governed by plant encoded disease resistance (R) gene products that upon recognition of pathogen encoded elicitor triggers downstream signal transduction cascades for rapid defense mobilization to inhibit pathogen growth (Bent and Mackey, 2007; Shirasu and Schulze-Lefert, 2000). Over the past two decades, more than 40 R-genes have been cloned from diverse taxa and have been grouped into five classes according to their structural characteristics (Dangl and Jones, 2001). Among the R-gene classes, the NBS-LRR constitutes the largest class (Meyers et al., 2003; Monosi et al., 2004) which has been further sub-divided into two based on the N-terminus domain. Thus those with a coiled-coil (CC) domain in the N-terminus constitute CC-NBS-LRR R-gene sub-class while those with an N-terminus TIR

*Abbreviations: ZzR*1, *Zingiber zerumbet* resistance gene 1; CC-NBS-LRR, coiled coil-nucleotide binding site-leucine rich repeat; rgcb, Rajiv Gandhi Centre for Biotechnology; MMLV, Moloney murine leukemia virus; Ct, cycle threshold; RT-PCR, reverse-transcription PCR; hpi, hours post infection.

* Corresponding author at: School of Biotechnology, National Institute of Technology Calicut (NITC), NITC Campus P.O., Calicut, Kerala, Postal Code-673 601, India. Tel.: +91 495 2285459, +91 9446404379; fax: +91 495 2287250.
E-mail address: aswati@nitc.ac.in (R.A. Nair).

0378-1119/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.gene.2012.12.011

domain with homology to intercellular signaling domains of *Drosophila Toll* and mammalian Interleukin-1 receptor constitutes TIR-NBS-LRR R-gene sub-class (Bent and Mackey, 2007; McHale et al., 2006). Structural specificity of the isolated R-genes has catalyzed the isolation of homologous sequences by candidate gene approach using R-gene specific degenerate primers, especially from those plants with a relatively large genome size (Leister et al., 1996) and/or wherein genetic studies have not been initiated so far. This approach has been successfully applied for the isolation and characterization of RGCs from many plant species (Aarts et al., 1998; Cordero and Skinner, 2002; Deng et al., 2000; Gedil et al., 2001; Hinchliffe et al., 2005; Lee et al., 2003; Li et al., 2006; Martinez-Zamora et al., 2004; Rivkin et al., 1999; Song et al., 1995). Some of these RGCs have been demonstrated to be linked to known R-genes (Leister et al., 1996; Shen et al., 1998). The RGCs have served as promising tools for the isolation of full-length resistance genes from many crop plants such as wheat (Feuillet et al., 1997), common bean (Ferrier-Cana et al., 2003), soybean (He et al., 2003) and citrus (Deng et al., 2000). Most of the isolated R-genes have been shown to exhibit constitutive expression (Grant et al., 1995; Hammond-Kosack and Jones, 1997; Song et al., 1995; Yoshimura et al., 1998) though their level of expression varies (Collins et al., 1999). However, some R-genes are induced by salicylic acid (SA) or pathogens, such as *XA*1 and *Pib* in rice (Wang et al., 1999; Yoshimura et al., 1998) and Hs1pro-1 in sugarbeet (Thurau et al., 2003). The constitutive expression is implicated in eliciting an immediate response in case of pathogen attack. Taxonomic relationships among plants govern the effectiveness of the isolated R-genes in imparting resistance to a heterologous background due to a phenomenon called "Restricted Taxonomic Functionality" (RTF) (Goggin et al., 2006; Tai et al., 1999). This necessitates that the R-gene be isolated from a species taxonomically close to the target species. So far no such effort has been carried out in any species of the family Zingiberaceae.

Hence towards the development of strategies for soft rot management in this important spice crop, candidate gene approach was followed to develop genomic tools to access the wild genetic resources that exhibit resistance to *P. aphanidermatum*. Following this PCR based approach, 42 authentic RGCs belonging to CC-NBS-LRR sub-class were cloned and characterized from *Zingiber* spp. (Aswati and Thomas, 2007a,b). The present research is focused on two important issues: (i). use of cloned *Zingiber* RGCs to obtain full-length sequences from *Z. zerumbet* and (ii). expression analysis of the isolated putative R-gene following *P. aphanidermatum* infection.

2. Materials and methods

2.1. Plant material

Z. zerumbet\ accession, rgcb2010-9 that is immune to the soft rot disease caused by *P. aphanidermatum* (Kavitha and Thomas, 2007) was used in the present study. The accession is being maintained at the germplasm collection nursery of Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala, India. Genomic DNA was extracted from young leaf tissue using GenElute™ Plant Genomic DNA extraction kit (Sigma, USA) following the manufacturer's instructions. The quality of the DNA was checked on a 0.8% agarose gel and purity by restriction digestion with *Eco*RV digestion.

2.2. Zingiber RGC specific primers for genome walker PCR

Four *Zingiber* RGCs designated — ZP2, ZP3, ZP5 and ZP9 (Accession numbers: AY864989, AY865006, AY864966 and AY865003) were selected for genome walker PCR analysis based on their constitutive basal expression pattern observed in earlier studies (Aswati and Thomas, 2007b). In order to identify the 5′ and 3′ region flanking the selected RGCs, oligonucleotide primers were designed (Fig. 1; Table 1) using the sequence information of selected *Zingiber* RGCs and custom synthesized with Sigma Genosys (Bangalore).

2.3. Isolation of flanking sequences of RGCs following genome walker strategy

Using the genomic DNA of *Z. zerumbet* restricted with *Eco*RV, *Dra*I, *Pvu*II or *Stu*I, four genome walker libraries were constructed with Universal Genome Walker kit (Clontech) according to manufacturer's instructions. The libraries were used as templates for primary and secondary genome walker PCR assays using *Zingiber* RGC gene specific (ZRGCgsp) primers and respective gene specific nested (ZRGCgspN) primers (Table 1) in combination with the adaptor primers provided in the kit according to the manufacturer's instructions using Advantage2 polymerase mix (Clontech) in Mastercycler eps (Eppendorf, Germany). The PCR cycling parameters for primary PCR consisted of an initial 7 cycles of 94 °C for 25 s and 72 °C for 3 min followed by 32 cycles of 94 °C for 25 s and respective annealing temperature (Table 1) for 3 min followed by a final extension at 67 °C for 7 min. For secondary PCR, the same program was followed except that instead of 32 cycles, the second step of PCR comprised of 20 cycles. PCR products were electrophoresed on a 1.5% agarose gel and amplicons eluted and gel purified using GFX PCR DNA and Gel Band Purification kit (Amersham Pharmacia).

2.4. Isolation of full-length Zingiber RGC genomic sequence

In order to amplify the full-length *Zingiber* RGC sequence, specific primers were designed (Table 1) based on sequences of 5′ and 3′ genome walker clones showing homology to R-genes. Following primary PCR, nested PCR was performed with the respective primers (Table 1) using 10 μl of 3/100 diluted first round PCR products using an Advantage PCR kit (Clontech) according to the manufacturer's instructions. PCR was carried out under the following conditions: initial denaturation at 95 °C for 5 min followed by 40 cycles of 95 °C for 30 s, 60 °C for 1 min and 72 °C for 5 min for the first PCR and initial denaturation at 95 °C for 5 min followed by 40 cycles of 95 °C for 30 s, 68 °C for 50 s and 72 °C for 5 min for the nested PCR. The amplified products were cloned and sequenced.

2.5. Sequencing and sequence analysis

Sequencing was carried out using BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA) cycle sequencing kit in an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Sequences were compared with those released in the GenBank database using BLASTX and BLASTP algorithms (Altschul et al., 1997). Multiple sequence alignments were obtained using ClustalX software with default parameters (Thompson et al., 1997) and based on these outputs, phylogenetic tree was generated using NJPLOT module of ClustalX software. Bootstrap analysis with 1000 replications was employed to evaluate the reliability of phylogenetic tree topology (Felsenstein, 1985). The theoretical isoelectric point and protein molecular weight and its probable cellular localization were estimated at EXPASY proteomic server. The CC structure was predicted by COILS (http://www.ch.embnet.org/software/COILS_form.html) and the presence of LRR repeats at LRRfinder (www.lrrfinder.com).

2.6. P. aphanidermatum infection and relative quantification by real-time PCR

Z. zerumbet (rgcb2010-9) was inoculated with freshly prepared *P. aphanidermatum* zoospore suspension (2.0×10^6 spores ml^{-1}) as described earlier (Kavitha and Thomas, 2007) at the collar region of each plant. The plants were maintained in a net house with misting facility, temperature between 27 and 30 °C and humidity between

Please cite this article as: Nair, R.A., Thomas, G., Molecular characterization of *ZzR*1 resistance gene from *Zingiber zerumbet* with potential for imparting *Pythium aphanidermatum* resistance in ginger, Gene (2012), http://dx.doi.org/10.1016/j.gene.2012.12.011