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# Isolation and molecular analysis of *R*-gene in resistant *Zingiber officinale* (ginger) varieties against *Fusarium oxysporum* f.sp. *zingiberi*

R. Swetha Priya, R.B. Subramanian *

*BRD School of Biosciences, Sardar Patel Maidan, Vadtal Road, Sattelite Campus, P.O. Box No. 39, Sardar Patel University, Vallabh Vidyanagar, 388 120 Gujarat, India*

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**Abstract**

Marker assisted selection (MAS) of resistant varieties is a reliable and faster method of selecting the right varieties for cultivation. The aim of the present study is to find the genes responsible for resistance in highly resistant varieties. In the present work we report the presence of a Resistance (R) gene of CC–NBS–LRR class of plant resistance genes. Both direct PCR amplification from genomic DNA as well as cDNAs, yielded a 0.6 kb DNA sequence indicating the absence of an intron. Sequence analysis of the PCR amplicon obtained from the genomic DNA showed very high homology to R-genes. An interesting observation from the present study is the presence of the R-gene in only resistant varieties. Neither the partially resistant or susceptible varieties showed the presence of this gene sequence. This in turn raises interesting questions on the evolution of these ginger varieties. The cloned R-genes provide a new resource of molecular markers for rapid identification of *fusarium* yellows resistant ginger varieties.


*Keywords:* CC–NBS–LRR class; *Fusarium oxysporum* f.sp. *zingiberi*; Marker assisted selection; RT-PCR; *R*-genes

## 1. Introduction

Ginger is an important spice crop and is also used in various medicinal and culinary preparations. *Fusarium oxysporum* f.sp. *zingiberi* (*Foz*), causing yellows is a serious pathogen of ginger in India (Stirling, 2004). To control and contain the spread of this fungus, suitable agronomic practices, varieties resistant to the pathogen and chemicals are used. Screening varieties is therefore important to select those suitable for *Foz* resistance and to identify the presence of resistance genes.

The success of a pathogen in infecting a host plant depends on how rapidly the plant recognizes the pathogen and activates appropriate defense reactions. If the pathogen carries an avr (avirulence) gene whose product is specifically recognized by the product corresponding *R* (Resistant) gene in the plant, resistance mechanisms are triggered rapidly resulting in disease resistance (Dangl and Jones, 2001).

Most plant R-genes that have been isolated to date encode proteins, which share structural similarities in that they have a nucleotide-binding site (NBS), and a leucine-rich repeat (LRR). Amongst plant species, NBS–LRR proteins can be further divided into two subgroups: those containing an amino acid terminus with homology to the *Drosophila* Toll and mammalian interleukin-I receptor (the TIR domain) which is essential for the development of the immune response and those which do not contain this domain, but often have it substituted with coiled–coiled (CC) domain. At present, three different *R*-gene-mediated signaling pathways have been described in *Arabidopsis thaliana*. The first involves the TIR–NBS–LRR type of *R*-genes (e.g. *RPP1* and *RPP5*) and requires

* Corresponding author. Tel.: +91 2692 234412; fax: +91 2692 236475/237258.
*E-mail addresses:* swethapriya@yahoo.com (R. Swetha Priya), bagsubbs@yahoo.com (R.B. Subramanian).

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*EDS1* (enhanced disease susceptibility) and *PAD4* (phytoalexin deficient) functions to attain full resistance. The second involves a subgroup of the CC–NBS–LRR type of *R*-genes (e.g. *RPM1* and *RPS2*) and requires functional *NDR1* (non-race-specific disease resistance) and *PBS2* (*avrPphB susceptible 2*). The third pathway involves the remaining CC–NBS–LRR type *R*-genes (e.g. *RPP7*, *RPP8*, and *RPP13*) and is independent of the function of *EDS1*, *PAD4*, *NDR1*, and *PBS2* (Dodds and Schwechheimer, 2002; Peart et al., 2002).

Disease resistance has not been yet characterized at the molecular level in ginger. Identification of specific genes involved in disease resistance not only provides a new source of molecular markers for rapid identification of fusarium yellows resistant ginger varieties but may also help in crop improvement programmes through genetic manipulation.

## 2. Methods

### 2.1. Ginger varieties and infection procedure

Infections were performed on eight varieties of ginger (*Zingiber officinalis*). Varada, Mahima, Rajetha, Maran, Himachal and Suprabha were obtained from Indian Institute of Spices Research, Calicut, Kerala. The two local varieties, GL1 and GL2 were obtained from National Research Center – Medicinal and Aromatic Plants, Boriavi, Gujarat. Tall (10–15 cm), 2 month old healthy potted plants were selected for *in vivo* bioassay. A volume of 5 ml of freshly prepared 10-day-old mycelial suspension which was grown on potato sucrose agar (PSA) at 28 °C was added to soil and the plants were watered and observed daily for 30 days. The pots were kept under shade and the severity of the disease was recorded after exhibition of symptoms on a scale of 0–4 (Baayen and De Maat, 1987). The experiments were repeated thrice. Stress related enzymes like catalase and peroxidase were also measured and the total amount of phenols, chlorophyll was estimated in all treated as well as control plants. The results obtained from the *in vivo* bioassay were confirmed through an *in vitro* bioassay on the same varieties (data not shown).

### 2.2. DNA isolation

Total nucleic acids were extracted from 1gm of fresh rhizome ground in liquid nitrogen in a pre chilled mortar and contents were incubated with extraction buffer (100 mM Tris–Cl (pH 8), 20 mM EDTA, 2 M NaCl, 4% CTAB, 0.3% β-mercaptoethanol, 1% PVP (w/v)) for 60 min at 60 °C followed by chloroform-isoamyl alcohol treatment for 15 min. DNA was diluted to 50 ng/μl final concentration in sterile deionised water; 2 μl of this solution were used in the amplification assay described below and stored in 1X TE buffer (10 mM Tris Cl pH 8, 1 mM EDTA pH 8) at −20 °C.

### 2.3. Primer design and PCR amplification for isolation of R-gene

Specific primers were designed using sequences obtained from ginger varieties from database. Degenerate primers were used for *R*-gene amplification [forward 5′ TGG TGG GGT TGG GAA GAC AAC G-3′; reverse 5′ TCC CGC TAG TGG CAA TCC CTA G-3′]. The reaction mixture contained 100 pM of primer, 200 μM of dNTP's, 0.25 Units Taq DNA polymerase approximately diluted with 10X Taq DNA polymerase buffer with 15 mM $MgCl_2$ and adjusted to a final volume of 12.5 μl with sterile milli Q deionised water. PCR was carried out in an Eppendorf Thermal cycler under the following conditions for 40 cycles: 94 °C for 30 s, 56 °C for 1 min, 72 °C for 1 min with an initial denaturation temperature at 94 °C for 1 min and a final extension at 72 °C for 5 min.

#### 2.3.1. Transformation, cloning and sequencing

R-gene products were excised separately from agarose gels and were extracted using Clean Gene I kit (Bangalore Gene I). The DNA sequence was ligated into a PTZ5R/T vector Inst/Aclone™ PCR product kit (Fermentas) in a vector: insert ratio of 1:3, competent *Escherichia coli* cells, strain JM109, were transformed and the clones were screened by blue/white colony selection (Sambrook and Russel, 2001). Plasmid DNA from the colony was isolated by the alkali lysis method (Sambrook and Russel, 2001). Recombinant DNA was screened for the appropriate size of the insert by digestion with EcoRI restriction enzyme. The cloned PCR product was sequenced using ABI 377 PRISM™ sequencer (Applied Biosystem, California) at Gene I, Bangalore, using SP6, T7 promoters for primer annealing.

### 2.4. RNA isolation and RT-PCR analysis

Total RNA was extracted from leaves of *Zingiber officinale* with extraction buffer (300 mM NaCl, 50 mM Tris–HCl (pH 8.0), 5 mM EDTA (pH 8.0), 2% (W/V) SDS, 10 mM β-mercaptoethanol) by incubating at 50 °C for 90 min. KCl (3 M) was added and incubated for 20 min on ice and was centrifuged at 5000*g*. Supernatant was collected and 8 M LiCl was added and incubated overnight to precipitate the RNA which was followed by the phenol extraction. NaCl (5 M) was added to supernatant and incubated overnight. RNA was dissolved in nuclease free water. To obtain cDNAs coding for *R*-gene, first strand cDNA was made from the above RNA samples with RT-PCR Kit (Bangalore Gene I) using M-MLV reverse transcriptase. cDNA was used as template using the above reaction mixture and PCR conditions.

## 3. Results and discussion

The disease symptoms like vascular wilt, yellows, root rot, and damping-off were observed within 3 days of infec-

Table 1
*In vivo* bioassay of eight varieties of ginger plants for resistance and susceptibility against *Fusarium oxysporun* f.sp. *zingiberi*

| Varieties | Plants [a] | Groups [b] | | | | |
|---|---|---|---|---|---|---|
| | Inoculated | 0 | 1 | 2 | 3 | 4 |
| Varada | 10 | Nil | Nil | 7 | 3 | Nil |
| Mahima | 10 | Nil | Nil | 6 | 4 | Nil |
| Rajesh | 10 | 5 | 5 | Nil | Nil | Nil |
| Maran | 10 | Nil | Nil | Nil | 4 | 6 |
| Himachal | 10 | Nil | 6 | 4 | Nil | Nil |
| Suprabha | 10 | Nil | Nil | 2 | 8 | Nil |
| GL1 | 10 | Nil | Nil | Nil | 7 | 3 |
| GL2 | 10 | 2 | 8 | Nil | Nil | Nil |

[a] Mean of the three experiments.
[b] 0 indicating no symptoms (resistant), 1 indicating slight symptoms such as one wilted leaf at the stem base or a brown discoloration of the stem base surface (resistant), 2 indicating a well developed characteristic unilateral wilt or otherwise still healthy plants (moderately resistant), 3 indicating severe wilt (susceptible) and 4 indicating a wilt death (highly susceptible).

tion with mycelial suspension. The symptoms were noted till 30 days. All the varieties exhibited variations in disease symptoms (Table 1). *In vivo* bioassay results correlated with biochemical tests and other bioassay results, previously accomplished. Rajetha, Himachal and GL2 varieties were highly resistant whereas Maran and GL1 varieties were highly susceptible.

A PCR product of 0.6 kb was obtained after amplification at 56 °C annealing temperature (Fig. 1). Sequencing of the 0.6 kb PCR amplicon yielded a readable sequence of 560 bp, which was compared for homology with sequences in the data bank in the NCBI website (http:/ncbi.nlm.nih.gov) using the BLAST programme. The highest homology was found with a pseudo *R*-gene and CC–NBS–LRR class of *R*-genes in ginger. The sequence was assigned accession no: EF 107542. RT-PCR performed at 56 °C with cDNA

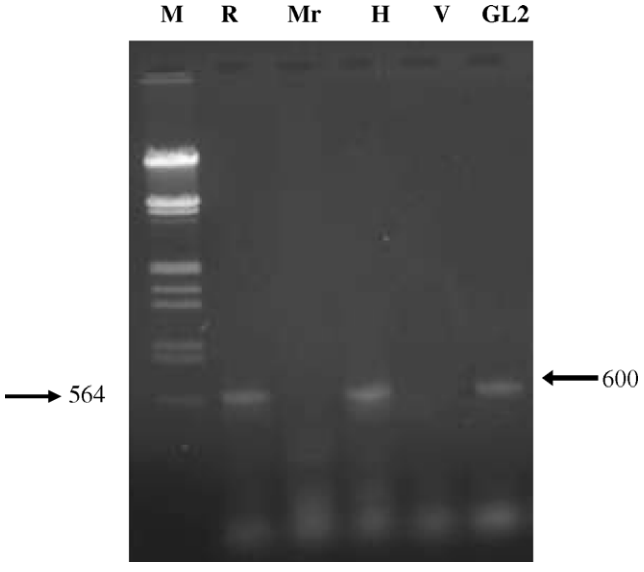


Fig. 1. Arrow indicates amplified PCR product of 600 bp only in highly resistant varieties Rajetha, Himachal and GL2.

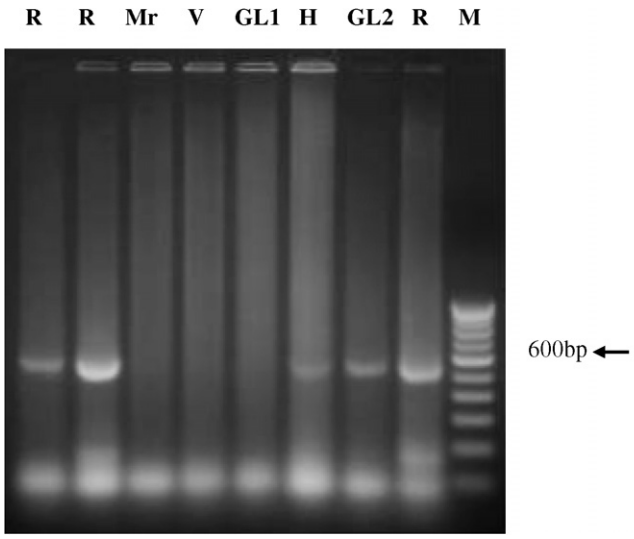


Fig. 2. RT-PCR analysis of *R*-gene using cDNA isolated from different ginger varieties. Arrow indicates presence 600 bp cDNA similar to the PCR amplified gene.

also showed a PCR product of 600 bp (Fig. 2), similar in size to the one obtained from direct PCR amplification from genomic DNA due to absence of an intron (Gen bank accession no: EF 209355).

Plant resistance genes often belong to large, clustered families. Proteins encoded by these host resistance determinants (*R*) are thought to interact directly or indirectly with cognate pathogen avirulence factors (*Avr*) in a gene-for-gene manner (Dixon et al., 2000). Large arrays of similar sequences allow for equal or unequal recombination events, resulting in the formation of several gene family members with new recognition specificities (Hulbert et al., 2001; Shunyuan et al., 2004).

The largest class of plant *R*-genes encodes proteins that contain a nucleotide-binding site (NBS) and leucine-rich repeats (LRR). About 150 genes predicted to code for NBS and LRR motifs are found in the genome of *A. thaliana* (Meyers et al., 2003) and they have been detected in most other plant species (Cannon et al., 2002). The broad occurrence of *NBS–LRR* genes in plants indicates an ancient origin, and this hypothesis is further supported by the homology of *NBS–LRR* gene products to animal proteins involved in the regulation of apoptosis and/or innate immunity (Dangl and Jones, 2001). However, it remains to be resolved more precisely when and how the *NBS–LRR* genes evolved into plant-specific *R*-genes It is interesting that the expression of *R*-genes was found only in highly resistant varieties like Rajetha, Himachal and GL2 but not in partially resistant or highly susceptible varieties. This raises the possibility that some classes of *R*-genes may have evolved recently in specific plant lineages via functional diversification of genes not originally specifying disease resistance.

Markers for disease resistance offer the additional advantage of permitting selection for resistance in the

absence of the pathogen or a variant of pathogen. The availability of PCR based markers for many disease resistant genes allows MAS for biotic resistance in plants to be successfully applied in any laboratory without the need of high technologies (Jansen, 1996). In addition, the rapid development of new molecular techniques, combined with the increasing knowledge on structure and function of resistant genes (Hulbert et al., 2001) will help in getting new molecular markers for MAS. In particular, the future perspectives for pathogen resistance in ginger would include: fishing of full-length sequence of R-genes and mapping of other resistant genes, especially for transferring QTL resistances.

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