Defence transcriptome profiling of *Zingiber zerumbet* (L.) Smith by mRNA differential display

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Soft rot is a serious disease in ginger (*Zingiber officinale* Roscoe), imposing a considerable economic loss annually in all ginger-producing countries. In this study, mRNA differential display was employed to identify genes whose expression was altered in a soft rot-resistant accession of *Zingiber zerumbet* (L.) Smith, a wild relative of ginger, in response to *Pythium aphanidermatum* (Edson) Fitzp., which is the principal causative agent of soft-rot disease in ginger. Analysis using 68 primer combinations identified 70 differentially expressed transcript-derived fragments (TDFs), of which 34 TDFs were selected for further analysis following reverse northern screening. Cloning and sequence characterization of the 34 TDFs yielded a total of 54 distinct clones. Functional categorization of these clones revealed seven categories, of which the defence/stress/signalling group was the largest, with clones homologous to genes known to be actively involved in various pathogenesis-related functions in other plant species. The significance of these genes in relation to the resistance response in *Z. zerumbet* is discussed. This study has provided a pool of candidate genes for detailed molecular dissection of the defence mechanisms in *Z. zerumbet* and for accessing wild genetic resources for the transgenic improvement of ginger.

[Kavitha P G and Thomas G 2008 Defence transcriptome profiling of *Zingiber zerumbet* (L.) Smith by mRNA differential display; *J. Biosci.* **33** 81–90]

1. Introduction

Ginger (*Zingiber officinale* Roscoe) is a herbaceous perennial belonging to the tropical monocotyledonous family Zingiberaceae. The thick, scaly, aromatic rhizome (underground stem) of the ginger plant is valued the world over both as a spice and as a medicine. India is the second-largest producer of ginger in the world, and contributed to one-fifth (230 000 metric tons) of the world production in 2005 (*http://faostat.fao.org/site/291/default.aspx*). Soft-rot disease caused by different species of the oomycete *Pythium* severely affects ginger cultivation in all ginger-producing regions of the world (Lawrence 1984). Once this soil-borne pathogen makes its appearance in a field, it spreads rapidly and kills the entire crop; moreover, the pathogen can survive in the soil for years as oospores. Infection begins at the collar region of the pseudostem of the ginger plant and spreads into the rhizomes, causing the inner tissues to decay, and ends in wilting and complete drying of the plant (Selvan *et al* 2002).

To date, no effective fungicides or biological control methods are available for controlling *Pythium* species (Folman *et al* 2003). Ginger is an obligate asexual reproducer, propagated exclusively through rhizomes. Conventional crop improvement methods involving sexual hybridization are therefore ineffective in ginger. Moreover, genetic variation for resistance to soft rot is not present in the ginger germplasm (Dake 1995). Given these impediments, transgenic improvement using a suitable genetic variation from alien resources could be a possible alternative for the genetic improvement of ginger. With that as a goal, we previously screened wild relatives of ginger for resistance to *P. aphanidermatum* (Edson) Fitzp., the most prevalent causative agent of soft-rot disease (Selvan *et al* 2002), and

Keywords. Differential display; ginger; *Pythium aphanidermatum*; soft rot; transcript profiling; *Zingiber zerumbet*

Abbreviations used: CaM, calmodulin; FGAM, formylglycinamidine; MAP, mitogen-activated protein; PCR, polymerase chain reaction; TDF, transcript-derived fragment

identified *Zingiber zerumbet* (L.) Smith as a potential source of resistance (Kavitha and Thomas 2007).

It is important to understand the network of genes that governs resistance to *Pythium* in *Z. zerumbet* in order to choose candidate genes for engineering soft-rot resistance into ginger. On the whole, the Zingiberaceae family is poorly studied and little is known about the molecular mechanisms of host resistance among its species. Though genetic determinants governing biotic and abiotic stress resistance have been reported in diverse plants (Kim *et al* 2000; Mahalingam *et al* 2003; Bray 2004; Ameline-Torregrosa *et al* 2006), extrapolating such results to taxonomically unrelated *Zingiber* species may not be appropriate, as the molecular events and biochemical processes vary between pathosystems (Ameline-Torregrosa *et al* 2006). Moreover, little is known about the molecular basis of host defence against oomycetes (Kamoun *et al* 1999; Latijnhouwers *et al* 2003), especially against the *Pythium* species, which affect mostly root tissues. We initiated a programme to understand the molecular mechanisms governing the resistance of *Z. zerumbet* to *P. aphanidermatum* and to further study the factors identified thereupon for the improvement of ginger. Here we report defence transcriptome profiling of a resistant accession of *Z. zerumbet* using the mRNA differential display technique to detect genes whose expression is altered in response to *P. aphanidermatum*.

2. Materials and methods

2.1 *Plant materials and pathogen inoculation*

A field isolate of *P. aphanidermatum* (RGCB P117) obtained from the Indian Institute of Spices Research, Calicut, Kerala, India was used for the inoculation studies. For zoospore production, mycelial discs were cut from the margins of a two-day-old *Pythium* culture and transferred to a Petri plate containing sterile double-distilled water and boiled pieces of grass leaves as spreading supports. The plates were incubated under direct light for 48 h. The plates with sporulating mycelia were given a cold shock at 4 °C for 10 min to release zoospores from the sporangia. A resistant accession of *Z. zerumbet* (Acc. no. 2010-9) (Kavitha and Thomas 2007) was used as the host. Rhizomes harvested from mature plants were sprouted in earthen pots in a red-earth:sand:leaf compost mixture and three-month-old plants were used for inoculation. The inoculation was done by pouring 500 μl of the zoospore suspension (2.0 x 10^6 spores/ml) onto the collar region (region just above the ground) of the pseudostem. Three pinpricks were given at the collar region before inoculation. The plants were maintained in a net house with a misting facility at 27–30°C and 80–90% humidity.

2.2 *RNA isolation and differential display*

For RNA isolation, tissue was collected from pseudostems inoculated with *P. aphanidermatum* zoospores at 24 h post-inoculation (hpi), 48 hpi and 96 hpi. Tissue collected just before inoculation (0 hpi) was used as a control. RNA was isolated according to Salzmann *et al* (1999). Total RNA was treated with DNase I to remove any traces of genomic DNA present. mRNA differential display analysis was carried out according to Liang and Pardee (1992) with modifications. Each RNA sample was subjected to three separate reverse-transcription reactions, each with one of the three one-base-anchored oligo-dT primers: $HT_{11}G$ (5′ AAGCTTTTTTTTTTG 3′), $HT_{11}C$ (5′ AAGCTTTTTTTTTTC 3′) and $HT_{11}A$ (5′ AAGCTTTTTTTTTTA 3′). Differential display analysis was performed on the reverse-transcribed products using the corresponding oligo-dT primers and random decamer primers (Operon Technologies, Alameda, USA). The random primers used in combination with each anchor primer are given in table 1. The 20 μl reaction medium contained 50 ng of reverse-transcribed product, 1X PCR buffer, 25 μM dNTPs, 2 μM random primer, 2 μM anchor primer, 0.2 μl of 2000 Ci/mmol α [^{33}P] dATP and 1 u Taq DNA polymerase (Qiagen). The polymerase chain reaction (PCR) conditions were as follows: 94ºC for 30 s; 42ºC for 1 min; 72ºC for 1 min for 25 cycles followed by 72ºC for 5 min in a thermocycler (Mastercycler gradient, Eppendorf).

2.3 *Gel electrophoresis, band elution and reamplification*

The differential display products were fractionated on a 6% denaturing polyacrylamide gel at 45 W for 2.5 h and the gel was autoradiographed. Differential bands were excised after aligning the autoradiogram with the gel. The excised gel pieces were rehydrated in 100 μl of sterile water for 10 min, and the swollen gel pieces were boiled for 15 min and centrifuged at 10 000 *g* for 2 min. The DNA was precipitated from the supernatant with double the volume of absolute alcohol in the presence of 60 mM sodium acetate and 100 μg/ml glycogen for 30 min at –70°C. The precipitate was rinsed with 85% ethanol and dissolved in 10 μl sterile water. The eluted fragments were reamplified following the same PCR conditions used for amplification of the reverse-transcribed product, but with non-radioactive dNTPs only. The eluted cDNA fragments were cloned using the pGEM-T Easy Vector System (Promega) and *E. coli* JM 109 as a host. Plasmid isolation from the transformed colonies was carried out using the Wizard Plus SV Miniprep DNA Purification System (Promega).

The PCR-amplified cDNA fragments eluted from the differential display gel were referred to as transcript-derived

fragments (TDFs) and the clones derived from the TDFs as cDNA clones.

2.4 *Reverse northern screening*

Reverse northern analysis was carried out on the differentially expressed TDFs and on clones derived from selected TDFs. For the preparation of Southern blots, 100 ng of reamplified TDFs or 100 ng of PCR-amplified recombinant plasmids were electrophoresed on agarose gels and blotted onto positively charged nylon membranes (Amersham Pharmacia) using a vacuum blotting apparatus (Amersham Pharmacia). For reverse northern hybridization, cDNA probes were synthesized from 1 μg of DNA-free RNA using a SMART™ cDNA synthesis and library construction kit (BD Biosciences) and were radiolabelled during the second-strand synthesis and subsequent PCR amplification.

For reverse northern hybridization, the blot was wetted with 2X SSC and pre-hybridized (0.5 M Na_2HPO_4 [pH 7.2], 2 mM EDTA, 7% SDS, 1% BSA and 10 μg/ml denatured salmon sperm DNA) at 65 °C for 4 h. A denatured probe was added to the pre-hybridization reaction and hybridization was continued for 16 h at 65 °C. Following hybridization, the membrane was washed twice with 50 ml 2X SSC and 0.1% SDS for 15 min each, and twice with 100 ml 1X SSC and 0.1% SDS for 15 min each at 65°C. The hybridized membrane was exposed to a phosphor screen and the exposed screen was scanned using a Phosphor Imager (BioRad). Densitometric analysis was performed on the hybridization signals using Quantity One software (BioRad). The values obtained from both the control and test hybridizations were normalized using the constitutively expressed housekeeping gene, *small ubiquitin-like modifier*, identified in *Z. zerumbet*.

2.5 *Sequence analysis*

Sequencing was carried out using an ABI 3730 automated DNA sequencer (Applied Biosystems, Perkin Elmer) using the ABI PRISM BigDye terminator cycle sequencing ready reaction kit, version 3.0. Homology searches were performed with BLAST and BLASTX algorithms using the nucleotide sequences against the non-redundant databases at the National Center for Biotechnology Information (NCBI).

3. Results

3.1 *RNA isolation and differential display*

Differential display profiles generated by 68 primer combinations (table 1) in four RNA samples, one from the control (0 hpi) and three from the *Pythium*-treated pseudostems (24 hpi, 48 hpi and 96 hpi), were examined visually for the distribution of each band across the samples.

Representative differential display profiles are shown in figure 1. Most cDNA bands were of similar intensity in both the control and *Pythium*-treated materials. These are likely to represent transcripts from housekeeping genes. However, some bands were unique to, or expressed in greater quantities in, either the infected or the control material. The former represent transcripts from upregulated genes whereas the latter represent those from downregulated

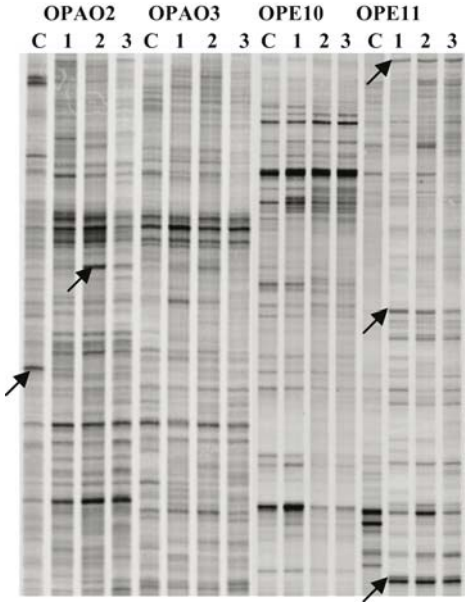

Figure 1. Differential display profile of RNA samples isolated at different times following *Pythium* infection from *Z. zerumbet* using the anchor primer $HT_{11}G$ in combination with random primers given at the top. Lanes: C – 0 hpi; 1– 24 hpi; 2 – 48 hpi; 3 – 96 hpi. Arrows indicate differential TDFs.

Table 1. Primer combinations used for mRNA differential display analysis of *Z. zerumbet*

Anchor primer	Random primer
$HT_{11}G$	OPC1,OPC3,OPC5,OPC6,OPE5,OPE10,OPE11,OPF1,OPF2,OPF5,OPG4,OPG5,OPG6,OPG17,OPJ1,OPJ5,OPJ6,OPJ7,OPAE1,OPAE2,OPAE3,OPAO1,OPAO2,OPAO3,OPAR1,OPAR2,OPAR3,OPAR5,HAP7,HAP8
$HT_{11}A$	OPA1,OPA2,OPA3,OPC1,OPC2,OPC4,OPE5,OPE10,OPE11,OPE13,OPF1,OPF2,OPF3,OPF10,OPF11,OPG1,OPG2,OPG3,OPJ2,OPJ3,OPJ4
$HT_{11}C$	OPF10,OPF11,OPF13,OPG7,OPG10,OPG13,OPAE4,OPAE5,OPAE6,OPAO1,OPAO4,OPAO5,OPAO6,OPAO18,OPAR4,OPAR5,OPAR6

genes. Altogether, 70 TDFs of >150 bp and representing both up- and downregulated genes were identified; all were eluted from the gel and purified for further analysis.

3.2 *Cloning, sequence characterization and reverse northern analysis*

The 70 TDFs were screened by reverse northern analysis using cDNAs pooled from the three test samples as a probe. Of the 70 TDFs, 36 hybridized with both the test and control cDNA probes yielding signals of almost equal intensity. These false positives were not analysed further. Of the remaining 34 TDFs, 17 showed clear differential signal intensities – again either unique to or expressed in greater quantities in the test compared with the control – and were categorized as 'reverse northern positives', while the other 17 gave no signals with both the probes and were grouped as 'reverse northern insensitives'. Both the reverse northern-positive and -insensitive TDFs were cloned, plasmids were isolated from four clones for each TDF and the inserts sequenced. Sequencing revealed that many of these TDFs contained more than one discrete sequence. From the 34 TDFs, 54 cDNA clones (ZP1–ZP54) with distinct sequence identity were obtained and subjected to sequence homology searches of the NCBI non-redundant databases – the results of these analyses are given in table 2 and described more fully in section 3.3. Of these, 35 were from reverse northern-positive TDFs and 19 from reverse northern-insensitive TDFs. All 54 cDNA clones were subjected to a second reverse northern analysis, again using cDNAs pooled from the three test samples as a probe. Twenty-one of the 35 clones derived from reverse northern-positive TDFs yielded detectable signals (figure 2a); the 14 remaining reverse northern-positive TDFs were undetectable, as were the 19 reverse northern-insensitive clones. Of the original 17 reverse northern-positive TDFs that showed differential signals, at least one clone of each yielded a detectable signal on a reverse northern blot.

The levels of transcripts of the 21 reverse northern-positive cDNA clones during disease progression were assessed by reverse northern analysis using the 24 hpi, 48 hpi and 96 hpi cDNA probes individually. Eight clones (ZP3, ZP4, ZP13, ZP15, ZP16, ZP21, ZP26, ZP29) were detectable only with cDNA probes made from RNA isolated at 24 hpi, whereas three clones (ZP6, ZP7, ZP51) showed a gradual upregulation from 24 hpi to 96 hpi (figure 2a, b). The signal intensities of five clones (ZP5, ZP9, ZP10, ZP28, ZP54) increased gradually up to 48 hpi but decreased at 96 hpi, whereas two clones (ZP30, ZP49) showed high intensities at 24 hpi, reduced intensities at 48 hpi, and higher intensities again at 96 hpi. ZP17, ZP22 and ZP43 showed a gradual reduction of signal intensities from 24 hpi, becoming undetectable by 96 hpi (figure 2a, b).

3.3 *Functional categorization*

Of the 54 cDNA clones (ZP1–ZP54), 70% could be classified into different functional groups based on their sequence similarities (E-value <1, score >40 and % homology >50%) to known genes/motifs (figure 3; table 2). Twenty-two genes (40.7%) comprised the largest class, the *defence/stress/signalling* category. Five genes (9.3%) were assigned to *protein synthesis*, and 3 genes each to *photosynthesis* (5.6%) and *general metabolism* (5.6%). Two clones each were assigned to the categories *post-translational modification* (3.7%) and *transcriptional regulation* (3.7%) and 1 clone (1.9%) to *developmental regulation*. Fourteen clones (25.9%) encoded polypeptide sequences with too little similarity to proteins of known function to be able to assign a function to them with confidence; we therefore classified them as having 'no significant homology'. Two clones (3.7%), also of unknown function, have apparent homologues in *Arabidopsis*. Although functional assignment based only on sequence homology needs experimental verification, it nevertheless provides a measure of the diversity of genes in the defence cDNA collection and helps to illuminate the defence pathways that play major roles in the pathosystem under study.

4. Discussion

4.1 *Differential display analysis*

The differential display technique employed in this study used RNA isolated from *Z. zerumbet* at different time intervals after *Pythium* infection to provide insight into the dynamics of the host response to the pathogen, including the complex signalling cascades activated during disease advancement (Rechsteiner *et al* 2006). Reverse northern hybridization was used to identify false-positive differentials resulting from PCR artifacts. However, because genes expressed at low levels may not yield detectable signals by blot hybridization, the reverse northern-insensitive TDFs were also cloned and sequenced along with the reverse northern-positive TDFs. This conservative approach permitted identification of clones such as ZP2 and ZP8 which are homologous to resistance protein-encoding genes expressed at extremely low levels and undetectable by RNA-blot procedures (Halterman *et al* 2003). Finally, the observed sequence heterogeneity of the differential TDFs has also been reported by others, in *Phaseolus vulgaris* (Torres *et al* 2006).

4.2 *Sequence annotation and functional categorization*

Of the seven functional categories defined, the *defence/stress/signalling* category was the largest (40.7%),

Table 2. The ID and size of the clones derived from differential TDFs from *Z. zerumbet* together with their sequence homology details

Clone ID	Size (bp)	Nature	Homology	Score (bits)	E-value	Percentage sequence homology
Defence/stress/signalling						
ZP1	600	DR	Putative quinone oxidoreductase	38.1	8.00E-03	52%
ZP2	300	UR	*Oryza sativa* clone sk13 NBS-LRR-like gene	56	5.00E-06	96%
ZP3	270	UR	Trypsin inhibitor gene	56	6.00E-06	96%
ZP4	400	UR	Ethylene insensitive protein 2	73.6	1.00E-13	81%
ZP5	450	UR	NADH-plastoquinone oxidoreductase chain	225	3.00E-59	88%
ZP6	200	UR	Ribonuclease-like PR 10 protein	40.1	2.20E-01	100%
ZP7	400	UR	Phosphoribosylformylglycinamidine synthase	89	4.00E-18	71%
ZP8	350	UR	*O. sativa* clone sk20 resistance protein	81.8	1.00E-13	100%
ZP9	500	UR	NAD(P)H-dependent oxidoreductase	110	8.00E-25	62%
ZP10	200	UR	Alternative oxidase	45.4	5.00E-05	90%
ZP11	300	UR	Proteasome beta subunit	56.2	2.00E-08	78%
ZP12	300	UR	Zinc finger (C3HC4 type zinc finger) protein	45.1	6.00E-05	68%
ZP13	700	UR	Pollen signalling protein with adenylyl cyclase activity	69.7	2.00E-12	68%
ZP14	500	UR	Calmodulin gene (CAM-3)	119	7.00E-25	100%
ZP15	400	UR	Protein kinase family protein	40	2.00E-03	62%
ZP16	400	UR	Histidine containing phospho-transfer protein	97.6	3.00E-18	98%
ZP17	200	UR	CTR-1 like protein kinase	42.1	7.00E-02	100%
ZP18	300	DR	Calmodulin (CAM-1) gene	56	5.00E-06	100%
ZP19	300	DR	G10 protein homologue	90.9	9.00E-19	97%
ZP20	400	UR	S receptor kinase PK3 precursor-like protein	71.9	1.00E-09	76%
ZP21	400	UR	Histidine kinase	98.4	6.00E-24	56%
ZP22	500	UR	Proline-rich cell wall protein	63.9	2.00E-10	63%
Photosynthesis						
ZP23	350	UR	Ribulose 1,5,biphosphate carboxylase	258	1.00E-65	94%
ZP24	300	UR	Chlorophyll a-b binding protein	110	1.00E-24	79%
ZP25	400	UR	Photosystem II stability/assembly factor	82	6.00E-16	88%
General metabolism						
ZP26	300	DR	Digalactosyl diacyl glycerol synthase I	42.4	4.00E-04	84%
ZP27	300	UR	Lipid transferase	40.8	1.00E-03	85%
ZP28	300	UR	Phospholipid/glycerol acyltransferase	81.6	5.00E-16	65%
Post-translational modification						
ZP29	300	UR	Chloroplast matk gene for maturase	93.7	3.00E-17	78%
ZP30	350	UR	Geranyl geranyl transferase subunit	99.6	6.00E-18	89%
Protein synthesis						
ZP31	270	DR	16S-like small subunit rRNA	244	1.00E-62	91%
ZP32	270	UR	18s rRNA gene	244	7.00E-63	91%
ZP33	700	UR	Mitochondrial ribosomal protein	161	2.00E-36	81%
ZP34	550	UR	Ribosomal protein gene	551	6.00E-154	98%
ZP35	300	UR	Eukaryotic initiation factor 4A (eIF4A)	40	6.00E-06	85%

Table 2. *(Continued)*

Clone ID	Size (bp)	Nature	Homology	Score (bits)	E-value	Percentage sequence homology
Transcriptional regulation						
ZP36	600	DR	RNA-binding glycine-rich protein	122	4.00E-28	78%
ZP37	400	UR	Putative chromosome condensation regulator	111	6.00E-25	68%
Developmental regulation						
ZP38	500	UR	ZFWD1 protein	182	2.00E-46	77%
No significant homology						
ZP39	270	UR	No significant homology	–	–	–
ZP40	212	UR	No significant homology	–	–	–
ZP41	600	UR	No significant homology	–	–	–
ZP42	150	UR	No significant homology	–	–	–
ZP43	300	UR	No significant homology	–	–	–
ZP44	290	UR	No significant homology	–	–	–
ZP45	750	DR	No significant homology	–	–	–
ZP46	250	DR	No significant homology	–	–	–
ZP47	300	UR	No significant homology	–	–	–
ZP48	200	UR	No significant homology	–	–	–
ZP49	180	DR	No significant homology	–	–	–
ZP50	180	DR	No significant homology	–	–	–
ZP51	280	UR	No significant homology	–	–	–
ZP52	450	UR	No significant homology	–	–	–
ZP53	500	UR	*A. thaliana*, unnamed protein product	100	8.00E-22	77%
ZP54	500	UR	*A. thaliana*, unknown protein	60.5	1.00E-09	65%

Clone ID of reverse northern-positive clones is in bold. Nature of the clones: DR, downregulated; UR, upregulated, is according to the nature of the respective TDFs in the differential display gel.

consisting of clones with homology to genes known to have roles during pathogenesis in other plant species (table 2). The possible functions of the genes placed in the other six functional categories are not obviously related to host defence, and a major proportion of the clones (29.6%) did not yield significant homology to sequences in the database.

The clones of the *defence/stress/signalling* category can be separated into two subcategories: (i) local and systemic signalling, and (ii) host defence, as follows.

4.2.1 *Clones with presumptive roles in local and systemic signalling:* Signal perception and transduction constitute one of the most important components of host resistance. Pathogen recognition is performed by resistance proteins encoded by R-genes (Dangl and Jones 2001). Two clones, ZP2 and ZP8, with homology to the R-genes of *Oryza sativa* were identified in this study and showed upregulation following *P. aphanidermatum* inoculation.

Calcium ion influx is one of the earliest events in challenged host cells following recognition of the pathogen, and calmodulin (CaM) plays an important role in sensing and transducing this event (Ali *et al* 2003). Plants have many CaM isoforms that are subject to differential regulation in response to external stimuli (Zhang and Lu 2003). In the present study, two clones with homology to CaM were identified, of which ZP14 was upregulated and ZP18 was downregulated.

Membrane receptors associated with G-proteins have been shown to be involved in defence signalling in rice (Suharsono *et al* 2002). The observed downregulation of ZP19, a G10-protein homologue, following *Pythium* infection of *Z. zerumbet* suggests two possibilities: the pathogen could be shutting down part of the signalling cascade, possibly to weaken the defence reaction of the host, or the host itself could be shutting down some of its non-essential processes to strengthen its defence reaction.

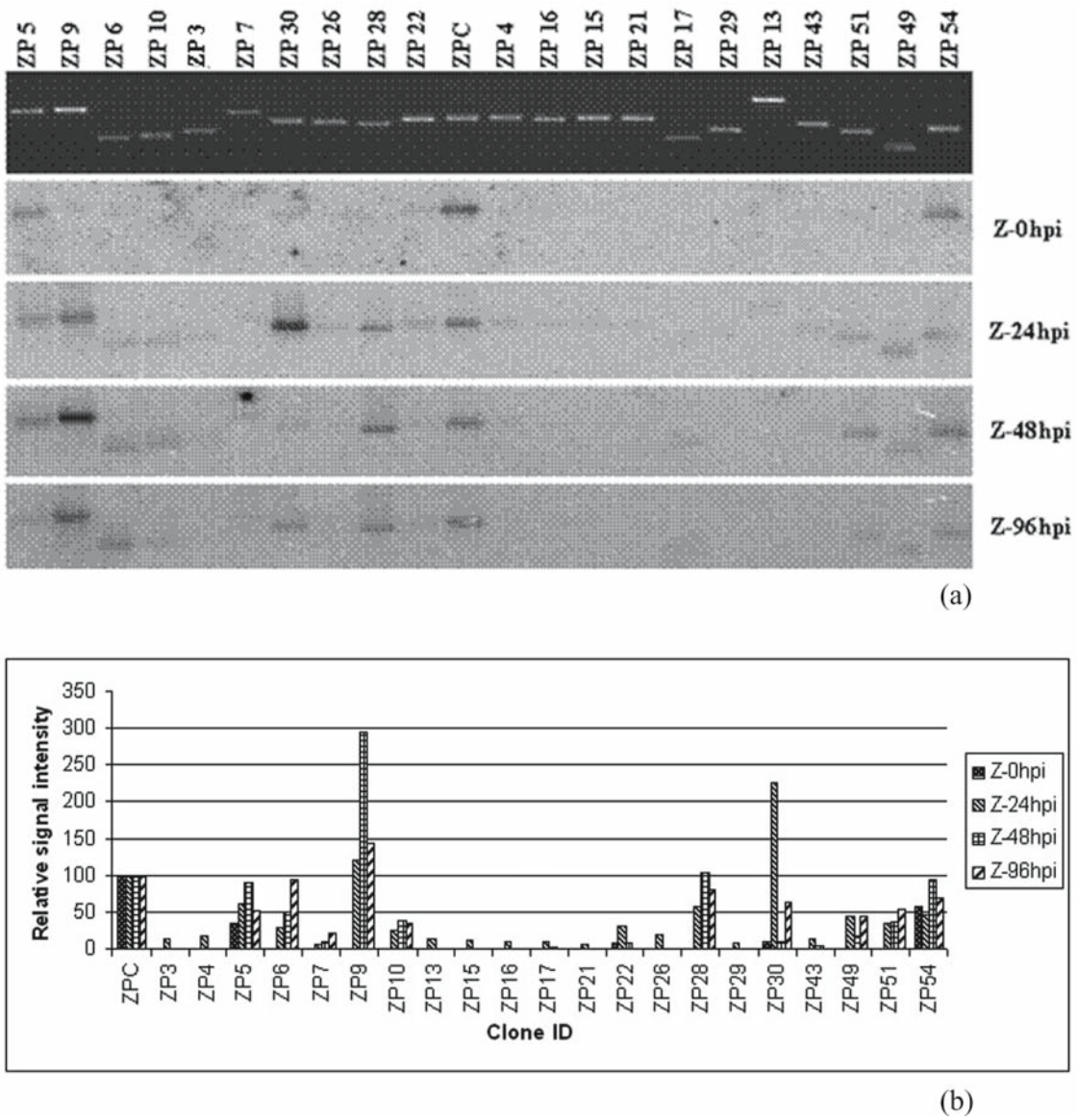

Figure 2. (**a**) Hybridization signal intensity of reverse northern-positive cDNA clones from *Z. zerumbet* following hybridization with labelled total cDNA synthesized from RNA samples isolated following *P. aphanidermatum* inoculation. The ethidium bromide-stained agarose gel used for blotting is shown at the top. Labels at the right side represent Z – *Z. zerumbet*; hpi – hours post inoculation. The clone IDs are as per table 2; ZPC – *Small ubiquitin-like modifier* used as internal control. (**b**) Relative signal intensity yielded by reverse northern-positive clones with cDNA probes from RNA isolated at different time intervals following *P. aphanidermatum* inoculation. Time intervals are given in the inset.

Kamoun *et al* (1999) suggest a general role for the hypersensitive response in plant resistance to oomycetes. ZP9, which registered a time-dependent increase in expression levels in the reverse northern analysis, encodes a polypeptide with strong homology to NAD(P)H oxidoreductase, an enzyme catalysing the formation of reactive oxygen species leading to the hypersensitive response (Papadakis and Roubelakis-Angelakis 2005).

Ethylene-mediated systemic signalling also plays a significant role in plant defence against oomycetes (Kamoun *et al* 1999). Five clones (ZP16, ZP21, ZP17, ZP4 and ZP15) with homology to various components of the ethylene-mediated defence-signalling pathway showed high levels of upregulation in *Z. zerumbet* immediately after *Pythium* inoculation, followed by a gradual reduction, as judged by reverse northern analysis. ZP16 and ZP21

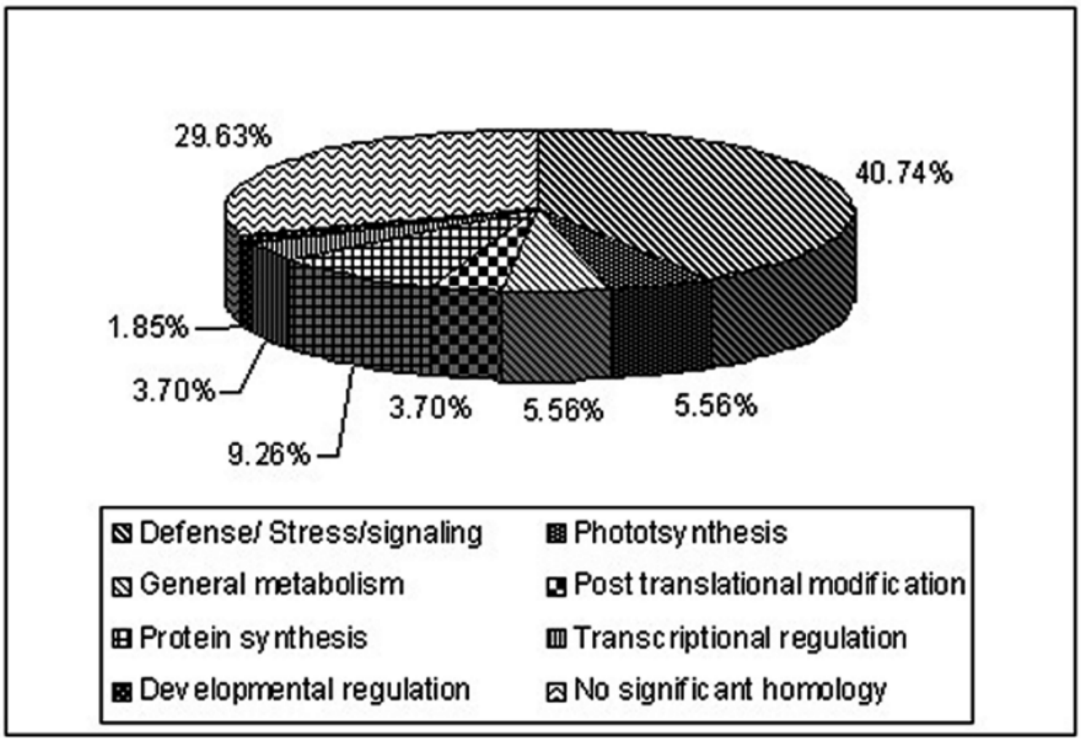

Figure 3. Functional categories of the 54 clones derived from the differential TDFs of *Z. zerumbet*.

specify proteins homologous to histidine kinases, the membrane-localized receptors of ethylene (Wang *et al* 2002). ZP17 shares homology with the *CTR1* gene, which functions downstream of the ethylene receptors and initiates mitogen-activated protein (MAP) kinase signalling in mammals (Wang *et al* 2002). Similarly, EIN2, with which ZP4 shares strong homology, is essential for signal propagation from CTR1 to the nucleus in *Arabidopsis* (Guo and Ecker 2004). Simultaneous upregulation of members of the ethylene signalling pathway at an early stage of *Pythium* infection suggests that this could be the most important defence signalling pathway in *Z. zerumbet* against *Pythium*.

The ubiquitin proteosome pathway, which has a key role in auxin-mediated cellular regulation, is important in the implementation of host defence in plants (Austin *et al* 2002; Tor *et al* 2003). The adenylyl cyclases are another important factor in auxin signalling in higher plants (Ichikawa *et al* 1997). The present study identified two homologues involved in auxin signalling: ZP11, homologous to the proteasome beta subunit gene, and ZP13, sharing homology with the adenylyl cyclase gene. Their upregulation in *Z. zerumbet* following *P. aphanidermatum* inoculation indicates the possible involvement of auxin-mediated defence signalling in this pathosystem.

4.2.2 *Clones with presumptive roles in host defence:* One of the downstream events following the hypersensitive response is the production of antimicrobial compounds (Osbourn 1996). Trypsin inhibitors, which are involved in inhibiting pathogen advancement (Chen *et al* 1999), are a crucial component in host defence. ZP3 is homologous to trypsin inhibitor genes, and it was expressed at elevated levels at 24 hpi, as determined by reverse northern analysis. Similarly, ZP6 shares homology with the ribonuclease-like PR 10 protein gene, a member of a group of pathogenesis-related genes encoding proteins with antimicrobial activity (van Loon and van Strien 1999). ZP6 showed a time-dependent upregulation following *Pythium* infection.

ZP5 encodes a polypeptide homologous to NADH-plastoquinone oxidoreductase. ZP5 transcript levels increased gradually following inoculation with *P. aphanidermatum*, reaching a recorded maximum at 48 hpi, and then declined by 96 hpi. The NADH-plastoquinone oxidoreductase of parasitic plants has an important role in suppression of the host immune response (Matvienko *et al* 2001a); in *Z. zerumbet*, the enzyme may detoxify substances produced by the pathogen. ZP1 has homology to a putative quinone oxidoreductase gene linked to the detoxification of phytotoxins in other pathosystems (Matvienko *et al* 2001b). Further studies are required to understand the significance

behind the downregulation of this reverse northern-insensitive clone.

ZP22 encodes a proline-rich polpeptide with homology to a protein involved in cell-wall strengthening. ZP22 is upregulated at 24 hpi, and its role in *Z. zerumbet* may be to inhibit entry of pathogens, as found in other pathosystems (Zhang and Mehdy 1994).

ZP10 is homologous to alternative oxidase, which protects the host from the overwhelming effects of its own reactive oxygen species generated during the host defence response (Bolwell *et al* 2002). Expression of this reverse northern-positive clone was maximal at 48 hpi in the reverse northern studies.

Clone ZP27 is homologous to the phosphoribosyl formylglycinamidine (FGAM) synthase gene of soybean. Reverse northern analysis of the corresponding gene revealed high expression at 24 hpi, reduced expression at 48 hpi, and elevated expression again at 96 hpi. The exact role of the FGAM synthase gene is not known; however, its expression is induced in soybean in response to nematode feeding (Vaghchhipawala *et al* 2004).

This is the first report of transcriptional changes in *Z. zerumbet* in response to the soft-rot pathogen, *P. aphanidermatum.* Using mRNA differential display analysis, we were able to identify cDNA clones with close homology to genes involved at different stages of defence in other plant species. Overall, the results strongly suggest a key role for ethylene-mediated defence signalling in the resistance response of *Z. zerumbet* to *P. aphanidermatum* infection. Such genes will be valuable for further deciphering the molecular basis of the resistance response and choosing suitable genetic variations to engineer *Pythium* resistance in ginger.

Acknowledgements

KPG gratefully acknowledges the Council for Scientific and Industrial Research (CSIR), New Delhi for the research fellowship (File No. 9/716(024)/2KI/EMR-I) and GT acknowledges the Department of Biotechnology (DBT), New Delhi for the research grant (Grant No. BT/PR2211/Agr/08/162/2001). The authors are thankful to the Indian Institute of Spices Research, Calicut for the fungal strain.

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MS received 1 May 2007; accepted 14 November 2007

*e*Publication: 12 January 2008

Corresponding editor: Renu Khanna-Chopra