31

Transcriptomic approaches for studying *Phytophthora* interactions in plants

George, K.J., Anandaraj, M., Kumar, I.P.V. and Vinitha, K.B.

INTRODUCTION

Black pepper (*Piper nigrum* L.) (2n = 52), originated in Western Ghats of India is the most important spice crop cultivated in India, Brazil, Indonesia, Malaysia, Sri Lanka, Vietnam, Cambodia and China. This spice with its characteristic pungency and flavour is an ingredient in many food preparations, as well as in medicines. Incidence of the dreaded *Phytophthora capsici*, is the major production constraint in all pepper growing countries. The impact of *Phytophthora* sp. on many economically important crops worldwide (Gregory, 1983) and in black pepper (Anandraj, 2000) has been well documented. Black pepper cultivars are susceptible to this pathogen and the degree of susceptibility varies among cultivars. *Piper colubrinum* (2n = 26), a distant relative of cultivated black pepper was found to be highly resistant to *Phytophthora* and is rarely exploited even though interspecific hybridization of this species with black pepper (Vanaja *et al.*, 2008) has been reported.

Conventional methods of gene cloning and sequencing are not only time-consuming and expensive but also yield only a limited amount of genetic information. However, next generation sequencing (NGS) methods, such as 454 pyrosequencing and illumina sequencing, provide a quick and easy means for either deep sampling or full sequencing of an organism's transcriptome that contains a very large number of expressed genes. NGS technologies thus have revolutionized transcriptomics by providing opportunities for multidimensional examinations of cellular transcriptomes in which high-throughput expression data are obtained at a single-base resolution (Morozova *et al.*, 2009) and advances in this technologies has resulted in the high throughput and low cost acquisition of EST reads from understudied species also (Vera *et al.*, 2008). Various applications of NGS technologies include transcriptome characterization, identification of novel transcripts/transcript isoforms, measurement of gene expression, identification of single-nucleotide polymorphisms and biological processes/pathways (Jain, 2011). The development of EST libraries not only from the plant studied, but also from the pathogen led to the concept of "interactome" (Birch and Kamoun, 2000), where the analysis of both plant and pathogen transcriptomes were combined. Illumina (Genome Analyser- GA IIx) is a short-read sequencing platform based on sequencing-by-synthesis principle and generates several million reads of desired length up to 150 bp. In the present study, we aimed to identify black pepper and *P. colubrinum* transcripts expressed in response to challenge inoculation with *P. capsici* utilizing illumina platform for next generation sequencing.

METHODOLOGY

Leaves of *P. colubrinum* and *P. nigrum* (CV. "IISR Shakthi" — tolerant to *Phytophthora*) were challenge inoculated with *Phytophthora capsici* and samples collected at various time intervals were pooled for mRNA isolation and sequencing. The sample preparation, sequencing and data analysis were done at Genotypic Technology, Bangalore, India. Essentially, the method involved mRNA isolation, fragmentation, cDNA construction, adapter ligation, and PCR amplification to create the final cDNA libraries. The cDNA library was sequenced (paired-end sequencing) using Illumina GA IIx, and the sequencing-derived raw image data were transformed by base calling into sequence data. The raw reads were cleaned by the trimming of adaptor sequences, empty reads and ambiguous nucleotides ('N' in the end of the reads). The reads obtained were then assembled using the SOAPdenovo program (Li *et al.*, 2010). The details of the RNA sequencing technique are as published by Nagalakshmi *et al.* (2010).

RESULTS AND DISCUSSION

Transcriptome sequence assembly and analysis was done to facilitate a system-wide approach to study *Piper-Phytophthora* interactions with special emphasis on the identification of genes involved in resistance to the oomycete. The assembly of sequence reads resulted in a total of 62619 and 101284 transcripts in case of *P. colubrinum* and *P. nigrum*, respectively. BLAST searches against plant (21 plant species including *Piper*), *Physcomitrella patens* (moss) and *Phytophthora* gene databases were utilized for similarity searches and assigning gene function. BLAST hits to mRNA and protein databases of different plant species and *Phytophthora* are given in Table 1. The significant hits were identified at different E-value cut-offs (out of 62619 transcripts from *Piper colubrinum* sample, 22921 transcripts were annotated and 42835 out of 101284 transcripts were annotated in case of *P. nigrum* sample).

Both *P. colubrinum* and *P. nigrum* transcripts showed maximum hit with *Vitis vinifera* (wine grape) sequences, followed by *Populus trichocarpa* (Poplar) sequences indicating closer relationship of magnoliids (order to which *Piper* belong to) with eudicots. Magnoliids are considered one of the largest clades of early diverging angiosperms and it is hypothesized that the magnoliids are sister to a large clade that includes both monocots and eudicots.

Table 1: BLAST analyses: Similarities of *P. colubrinum and P. nigrum* transcriptome sequences against different plant species and *Phytophthora* sequence database

Target organism	Number of annotated transcripts *P. colubrinum* (mRNA and protein)	Number of annotated transcripts *P. nigrum* (mRNA and protein)	Total annotated transcripts (from both the species)
Arabidopsis thaliana (thale cress)	14334	27998	42332
Brachypodium distachyon (purple false brome)	217	293	510
Brassica napus (oilseed rape)	654	1128	1782
Carica papaya (papaya)	493	666	1159
Glycine max (soybean)	9511	19204	28715
Gossypium hirsutum (cotton)	1576	2883	4459
Hordeum vulgare (barley)	1620	3142	4762
Lotus japonicus (lotus)	3267	6043	9310
Manihot esculenta (cassava)	403	600	1003
Medicago truncatula (barrel medic)	4653	9531	14184
Mimulus guttatus (mimulus)	25	-	25
Oryza sativa (rice)	3981	7389	11370
Persea americana (avocado)	187	304	491
Physcomitrella patens (moss)	764	1338	1902
Phytophthora sp.	738	1432	2170
Piper sp.	152	117	269
Populus trichocarpa (poplar)	17558	33774	51332
Ricinus communis (ricinus)	403	32418	32821
Solanum lycopersicum (tomato)	7855	15367	23222
Sorghum bicolor (sorghum)	13339	25082	38421
Triticum aestivum (wheat)	6294	12149	18443
Vitis vinifera (wine grape)	19515	37105	56620
Zea mays (corn)	12662	20486	33148

Identification of genes involved in *Piper – Phytophthora* interactions

Gene ontology assignment programs for functional categorization of those annotated unigenes were done based on similarity with *Arabidopsis* sequences. In the case of *P. colubrinum* transcripts, 3160 were characterized under molecular functions (GOMF), 5866 under biological process (GOBP) and 2893 under cellular components (GOCC) category. Similarly, *P. nigrum* transcripts were able to map 3469 to molecular functions, 6549 to biological processes and 3419 to cellular component category. The genes involved in other important biological processes such as response to abiotic and biotic stimulus/stress, transport, transcription and signal transduction, were also identified through GO annotations. Broadly, the putative orthologs of genes involved in various pathways and cellular processes were found in both the transcriptomes. The identified stress induced genes include catalase, chitinase class I and VII, glutathione-S-transferase, peroxidase, beta 1,3-glucanase, Cu/Zn superoxide dismutase, manganese superoxide dismutase, MAP kinase, osmotin etc. Among the genes, those identical to genes involved in secondary metabolism were, chalcone isomerase, chalcone synthase, cinnamate 4-hydroxylase, cinnamoyl-CoA reductase, geranyl geranyl pyrophosphate synthase, hmg-CoA reductase, lycopene beta cyclase, phenylalanine ammonia lyase, p-coumaroyl shikimate 3'- hydroxylase and transaldolase. A variety of transcription factors and genes involved in primary metabolism with significant similarity to those characterized in other plants were also identified in both transcriptomes. We are currently analyzing the expression patterns of many of these important genes for their characterization and possible use in imparting resistance to *Phytophthora* in black pepper. The expression of specific pathogenesis related proteins in black pepper in relation to *Phytophthora* infection was also studied by Nazeem *et al.* (2008), who confirmed the role of β-1, 3 glucanase and related enzymes in the defense mechanism of black pepper against foot rot disease.

In most cases, resistance genes (R genes) are of the nucleotide-binding domain and leucine-rich repeat (NBS-LRR) class (Caplan *et al*., 2008), encoding receptor-like proteins that most likely recognize an avirulence factor and trigger a defense response. Resistance Gene Analogs (RGAs) share several common motifs that are highly conserved. These include the P loop (phosphate-binding domain), the kinase-2 motif, and the GLPL motif. These motifs have been widely utilized for the identification or the cloning of resistance genes. Nine NBS related transcripts from *Piper colubrinum* were found and they were related to sequences of *Arabidopsis thaliana, Populus trichocarpa, Brassica napus, Glycine max* and *Hordeum vulgare*. Similarly, about 15 transcripts from *Piper nigrum* was found to be related to NBS type of resistance genes. Transcription factors (TFs) represent key proteins that bind to specific DNA sequences and regulate gene expression. TFs are represented by various multigene families and are highly conserved in eukaryotic organisms, especially plants. A large number of sequences with similarity to various TF genes identified in plants were discovered from both the transcriptomes.

Expression of *Phytophthora* genes *in Planta* were also examined and when both transcriptomes were considered together maximum number of genes were from *Phytophthora infestans*, followed by *P. capsici*. This could be because maximum annotated gene list is available in case of *P. infestans*, compared to that of *P. capsici*. Limited number of genes from *P. palmivora* and *P. tropicalis* were also annotated. Some of the matching sequences from different *Phytophthora* species were, for catalase, alfa and beta-tubulins, heat shock proteins, calcium/calmodulin dependent protein kinase 3, enolase, endo-1, 4-beta glucanase, ubiquitin family proteins, Rab1 family GTPase, calmodulin and members of the Ras super family of monomeric GTP-binding proteins, essential in specific steps of vesicle transport and secretion.

Improvements in crop productivity require adoption of new breeding technologies. Integration of genomic and transcriptomic data provides an opportunity to generate newer molecular resources for improved breeding technologies and crop improvement. Transcriptome sequencing is one of the most important tools for gene discovery. Consistent with other publications, these results demonstrated that illumina paired-end sequencing can successfully be applied to non-model organisms. *P. colubrinum* is one useful species as a resistance source against *Phytophthora* and is presently used with black pepper mainly as a rootstock. Besides being highly resistant to the foot rot causing *P. capsici* it is also found resistant to nematodes that cause root knots in black pepper. Earlier efforts on suppression subtractive hybridization by Batista de Souza *et al.* (2011) to identify differentially expressed sequences in roots of black pepper infected by *Fusarium solani* f. sp. *piperis* and studies in *P. colubrinum* has resulted in the identification of sequences coding for putative proteins related to oxidative burst and defense response. Studies by Dicto and Manjula (2005), Mani and Manjula (2010) in *P. colubrinum* also resulted in the identification

of a set of candidate defense genes. The transcriptome data developed through this study is expected to provide the foundation for research on gene expression, genomics and functional genomics in black pepper.

SUMMARY

Transcriptome sequencing provides an overview of the genes expressed in particular cell types and developmental stages in host and pathogen and allows sampling of genes expressed as part of the interaction transcriptome. In order to survey genes associated in plant-pathogen interactions, RNA was extracted from *Piper colubrinum* and *P. nigrum* leaf tissues challenged with *Phytophthora capcisi*. RNA sequencing was done using Illumina (Genome Analyzer II) paired-end technology, each sequencing feature yielding 2 × 72 bp independent reads from either end of a DNA fragment. Analysis of transcriptome data revealed expression of many stress induced genes as well as genes related to secondary metabolism. A variety of transcription factors and genes involved in primary metabolism with significant similarity to those characterized in other plants were also identified. The resistance and defense related genes identified in the study provides many new candidate genes for developing resistance to *Phytophthora*.

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