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Expression Profiling of Bioactive Genes from *Moringa oleifera*

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Abstract Plants are under constant assault by biotic and abiotic agents. When an elicitor is prologued, an immense reprogramming of plant gene expression and defense responses are initiated, which could be a natural source for potential drug development and insertional mutagenesis. In this regard, differential expression analysis of a medicinal plant *Moringa oleifera* was performed for bioactive genes at seedling stage, using differential display-RT-PCR technique. Infected seedlings with a fungus *Fusarium solani* collected at different time intervals, showed a massive change in their gene expression profile. The data analysis revealed that at least 150 pathogen-induced and about 60 suppressed genes were differentially expressed at 8-h postinoculation of the biotic stress. Fifty-five selective genes were disunited and reamplified. Sequence analysis of these potential genes illustrated that these genes had properties of some induced peroxidase mRNA, cell proliferation, others were mitogen activated protein kinases, ribosomal protein genes, defense regulating genes, and a few also had structural properties. Further studies about the utility of these genes in plant metabolism could assist to develop improved transgenic breeds with enhanced value of infection tolerance not only of *M. oleifera* but of other cultivars also.

Keywords *Moringa oleifera* · *Fusarium solani* · DDRT-PCR · BLAST · Bioactive genes

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Introduction

During the past few decades, curiosity in antifungal and antimicrobial compounds from medicinal plants has been expanded due to the improved resistance of fungal pathogenesis to currently employed antifungal drugs and the toxicity or adverse reactions of the anti-infective [1]. A precise study on plant immunity and in cataloging the pathogen-infection strategies has cleared the picture of plant-pathogen interaction [2]. The most familiar and diverse stimulant among biotic assaults are fungal species with a numeral of more than 8,000 disease-causing species [3]. In fungal infection, one of the mechanisms comprises the synthesis of low molecular mass inhibitory compounds with the aptitude of acting directly on fungi to obstruct growth [4]. Plants with fungal molest are always hauler of fungal diseases, which may result in expression of huge array of bioactive genes either inducible or constitutive manner. There is also a great deal of up- and downregulated genes after fungal stimulation. These genes and newly induced genes could be potentially paramount source to study tolerance mechanism in plant and to introduce fungal-resistant breeds [5].

Moringa oleifera is one of these most thriving plants having antimicrobial properties in different extracts of it [6] and is vulnerable to *Fusarium solani. Moringa* used in the customary medicine passed down for centuries in many cultures like Unani-Tibb/Greco-Arab and Ayurveda system of medicine. *Moringa* has antibiotic, antitrypanosomal, hypotensive, antispasmodic, antiulcer, anti-inflammatory, hypocholesterolemic, and hypoglycemic activities, as well as having considerable efficacy in water purification by flocculation, sedimentation, antibiosis, and even reduction of Schistosome cercariae titer.

The corresponding plant defending gene could be induced and isolated after treatment of plant with the fungus [7]. In our project, we stepped to isolate such type of genes from *M. oleifera* after fungal stress by *F. solani* using differential display PCR (DDRT-PCR) which is a very useful technique for identification of differentially expressed genes by comparative display of arbitrarily amplified complementary DNA (cDNA) subsets [8]. In a literature appraisal of DDRT-PCR-based expression profiling of different medicinal plants genes, *Moringa* gene expression profile could not be found under stressed condition of a fungus, *F. solani*. The expression outline of induced and noninduced seedlings collected at different time intervals exhibited a differential response using a limited set of primer combination [9, 10]. The amplicons were related to the properties of induced peroxidase messenger RNA (mRNA), cell proliferation, mitogen-activated protein kinases, ribosomal protein genes, defense regulating genes, and a few also had structural properties. The results showed that the differential display procedure possessed enough sensitivity to be applied to the detection of fungal genes induced during a plant-pathogen interaction. On these bases, we can conclude that *M. oleifera* has a great potential to be used as alternative drug formulation and development of resistant cultivars.

Materials and Methods

Seeds of Medicinal Plant

Seeds of *M. oleifera* procured from the main markets of Shahkot (Sheikhupura) and Faisalabad were taxonomically identified and confirmed from the Department of Botany, University of Agriculture, Faisalabad, Pakistan.

Induction of Seeds and Sample Collection

The seeds first lead to induction by pathogenic fungus *F. solani*. Seeds without induction were used as control [7]. Samples were collected on the basis of specific time interval of 0, 8, 16, and 24-h postinduction under control environment of temperature, light, and humidity. Samples were stored at −80 °C till further analysis [11, 12].

DDRT-PCR

Total RNA was derived from control (normal) and sample (differentially treated) seeds by using RNA isolation kit (QIAGEN). RNA was incubated with RNase inhibitor and RNase-free DNaseI to remove contaminating DNA [13].

The differential display PCR was performed after Falak and Jamil [5]. Briefly, the RNA was converted into cDNA by reverse transcription using an oligo-dT primer (anchored primer) from 5′ HTTTTTTTTTTTTM (where M=A, G, or C; H=AAGC).

All PCRs were done in doublets using arbitrary primer series, HAP-25 to HAP-32. The PCR profile for differential display was set as follows: 94 °C for 30 s, 42 °C for 1 min, 72 °C for 30 s, 40 cycles, followed by a 5 min final extension at 72 °C The PCR products were resolved on 6 % denaturating polyacrylamide gel using 8 M urea and 1× TBE at 100 V and silver stained. The bands for up- and downregulated genes were excised from the gel and reamplified using the same PCR conditions except that the concentration of mixed dNTPs was twofold increased to 0.02 mm. The reamplified products were sequenced from the DNA sequencing facility of the Center for Applied Molecular Biology, Lahore, Pakistan, under the Access to the Scientific Instrumentation Program of the Higher Education Commission of Pakistan.

BLAST Analysis

The homology of the results obtained after DNA sequencing was analyzed using Basic Local Alignment Search Tool (BLAST). The similarities with other sequences from different plants were determined.

Results

Gene expression profile of a medicinal plant *M. oleifera* was studied under a fungal stress. Seedling growth conditions such as temperature, light, and humidity were optimized in an artificial climate chamber. The seeds were germinated in Petri plates, and seedlings were observed after 2 to 3 weeks.

Seedlings of *M. oleifera* were inoculated with a fungus *F. solani* at the fifth day of seed germination. The control and inoculated samples were harvested after the fifth day of germination with a duration of 8 h and total RNA was extracted from each sample (Fig. 1).

Harvesting of seedlings and isolation of RNA at certain time intervals was selected on the basis of previous studies. The expression level of defense genes is synchronized at transcript level in infected plants [14]. According to Falak and Jamil's depiction, these transcripts initiate to accumulate 1 h after stimulation of plant, but ceiling level is achieved according to the condition and compatibly of fungus to a plant [5]. Actually, defense transcriptomes are also greatly influenced by compatible, noncompatible, and symbiotic relation of fungus to host plant [15], e.g., if pathogen is noncompatible to the host then it will accelerate more number of

Fig. 1 A representative figure showing total RNA extracted from *Moringa oleifera*: 1.2 % agarose gel electrophoresis showing total RNA isolated by QIAGEN kit method from *F. solani*-infected seedlings; *M* is the 1-kb DNA ladder (Fermentas), *L1*, *L2* represents second and third fungal-treated samples

defense genes to accommodate the situation as compared to compatible and symbiotic ones; besides, about 8,000 phytopathogenic fungal species are reported [16]. So the collection of infected and noninfected samples and hence mRNA at different time intervals may give us a clear picture of defense-gene induction phenomena. In our case, on the basis of total RNA quantification and agarose gel analysis, the maximum transcript level was achieved at 8 hpi (hours post inoculation). Mohr et al. [17] found that transcripts begin to accumulate 12- to 24-h postinduction and about ten times increased level in infected roots as compared to the control samples in common bean was observed with *F. solani* induction The defense gene response of a plant at early stages of infection is mostly due to beginning of the ailment after which fungus may maintain its growth by overcoming the defense genes [5].

Differential Display-PCR

The infected samples with maximum total RNA concentration and their respective controls were selected for the comparison of induction or repression of antifungal genes in *M. oleifera*. Following purification, RNA was primed in the first-strand synthesis with the hybrid oligo (dT) linker primer [18]. This cDNA was used for the PCR reaction [19].

DDRT-PCR technique was applied to the selected samples with specific set of anchored and arbitrary primers; amplicons were separated on silver-stained denaturing gel. Some representative results of denaturing page after PCR with different arbitrary primer are given in Fig. 2.

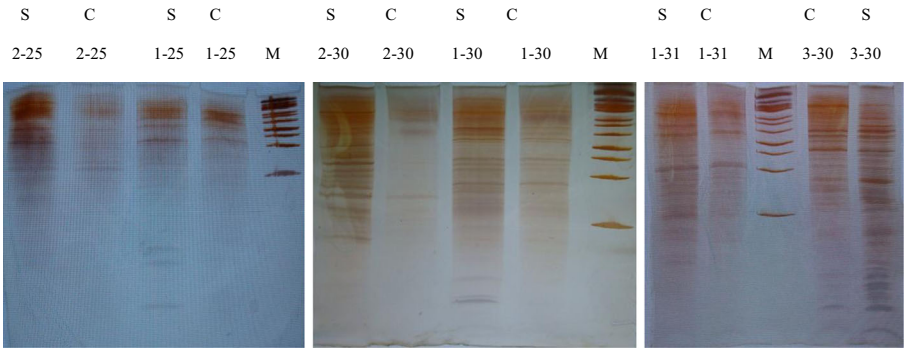

Fig. 2 Example of DDRT-PCR of *M. oleifera* seedlings from control and fungal-induced samples (8 hpi) with arbitrary primer HAP-26 and anchored primer HT11G and b with arbitrary primer HAP-26 and anchored primer HT11C. PCRs were performed as described in the text. Amplified cDNA fragments were resolved by denaturating gel electrophoresis. *Arrows* indicate the differentially expressed cDNA fragments that were recovered from the gels and analyzed further

In 24 sets of reactions, we found 150 pathogen-induced genes, and about 60 genes were suppressed at 8 hpi of the biotic (fungal) stress. Since there is a great chance of false-positive results in such experiments [20], therefore sequencing of the genes could confirm us the reliability of these differentially expressed genes. The results are shown in Fig. 3 with differentially expressed genes indicated by arrows.

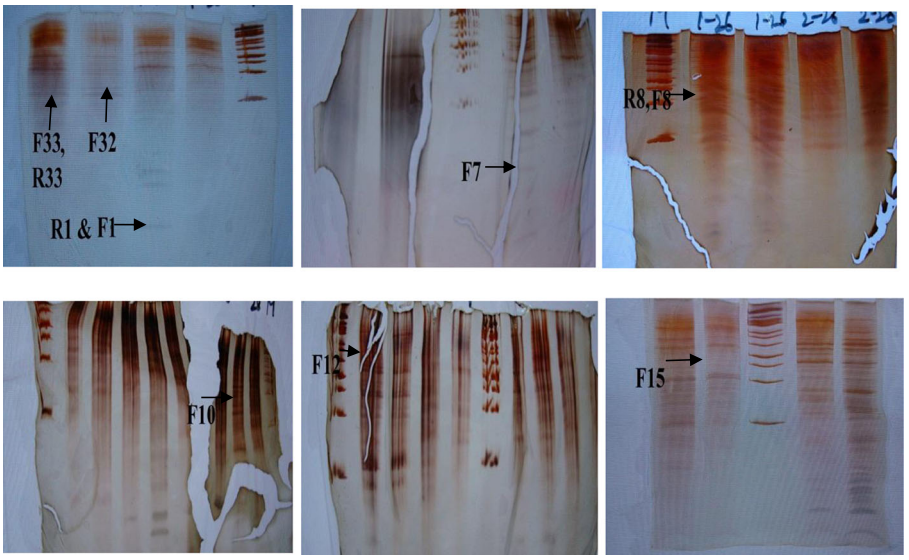

Fig. 3 Some representative gels showing differential expression of genes from fungal-induced seedlings of *Moringa oleifera*. The seedlings were inoculated with *Fusarium solani* and harvested at different time intervals. RNA was isolated followed by DDRT-PCR. The samples were run on Urea-PAGE and silver stained. Differentially expressed bands with significance are marked with *arrows*

BLAST Analysis

Because no report on differential fragments from *M. oleifera* on gene sequence analysis under stress conditions could be found, therefore, a very low output of *M. oleifera* differential fragments was observed by sequence alignment and homology searches in finding significant similarities with known sequences of other plants. For discovering genes whose expression has been altered under particular conditions, the DDRT-PCR, however, does not require prior knowledge of genomic information of the species of interest [5].

Out of the 55 upregulated reamplified cDNA products, 25 fragments showed significant homologies with the genes that are involved in known biological processes (Table 1) and the sequence homologies varied from 90 to 14 %. To consider the involvement of differential products in putative biological functions, the homology range is enough. Because for 200–100 query length, the sequences having 20–25 % identity are considered as homologous and most likely to be involved in biological functions related to known ones. The consideration gets strengthened especially with low E values for shorter DD fragments that minimize the similarities of the sequences on accidental basis [21]. Generally, the differential cDNA bands showed homology with bacterial-induced peroxidase, rpl16, rab GDP dissociation inhibitor alpha-like, 60S ribosomal protein L14, and AtPSK1 genes (Table 1).

Discussion

M. oleifera is a medicinal plant having antimicrobial properties [6, 8, 22]. The antimicrobial activity of the extracts of *M. oleifera* affected predominantly bacterial species. Shahid et al. [23] concluded that *M. oleifera* was a good source of antimicrobial proteins. A 14.4 kDa peptide was purified from the plant that exhibited potent and broad-spectrum antimicrobial activity. Such antimicrobial proteins and other proteins related to plant defense can be induced by infecting the seed by some fungus [5, 24, 25].

Hence, after reviewing the medicinal properties of *M. oleifera*, it was selected to the study differential expression of bioactive genes under fungal stress.

Pathogen attack initiates a cascade of signals transduction pathway, so that systemic or induced resistance begins in tissue remote to the initial infection [26]. Defense responses to different biotic assaults are extremely variable in different plants because of the unique co-evolutionary relationships between specific plant species and the specific agents of damage [15].

Plants show activation of different resistance mechanisms against fungal attack and a comprehensive evaluation of changes in the expression of these resistance genes needs to be addressed to understand the molecular perspectives of these mechanisms. For the screening of changes in expression profiles of normal and fungal-induced mRNAs (Table 1), DDRT-PCR was found to be successful. Twenty-four cDNA amplicons showed significant homologies with the genes involved in known biological processes such as translation, defense mechanisms, and metabolism. The roles of these amplicons are discussed here.

In the expression of resistance in plants, the key early event is the oxidative burst infected by incompatible pathogens [27]. cDNA amplicons F7 and R8 correspond to bacterial-induced peroxidase gene. Peroxidases (POD) (EC 1.11.1.1.7) which are known to be activated in response to bacterial or pathogens attacks are a group of heme-containing glycosylated proteins. Our finding also coordinates with the studies that in host-pathogen interactions [28] several roles of plant POD have been recognized including xanthomonad-induced resistance [29, 30]. The association of bacterial blight with the activity of POD was suspected

Table 1 Differentially expressed cDNAs isolated from *Moringa oleifera* under *Fusarium solani* induction

cDNA	Upregulation	Gene identified	Biological function	Accession number	Query age (%)	*E* value
F1	Significant	*Solanum lycopersicum* uncharacterized LOC101266876 (LOC101266876), mRNA	Uncharacterized protein	XM004231150	14	0.008
F7	Slight	*Gossypium hirsutum* bacterial-induced peroxi dase mRNA, complete cds	Defense against pathogen or insect attack	AF155124	40	1e-13
F8	Slight	*Brassica oleracea* var. italica isolate ML11_36 rpl16 gene, partial sequence; chloroplast	Cell proliferation	AY752711	34	4e-17
F10	Moderate	*Pitcairnia carinata* isolate Crayn 6 rpl32-trnL intergenic spacer, partial sequence; chloroplast	Intergenic spacer in the small single copy (SSC) region of the chloroplast genome	HQ913793	35	0.69
F12	Slight	*Cucumis sativus* clone Tcs 3 retrotransposon Ty1-copia, complete sequence	Evolutionary studies	EF122143	13	0.16
F15	Slight	*Populustrichocarpa* glucose-6-phosphate de hydrogenase (G6PDH2) gene, complete cds	Metabolism	DQ343567	17	3.4
F20	Significant	*Arabidopsis lyrata* subsp. lyratabZIP transcription factor family protein, mRNA	Interactions between bZIP transcription factors play important roles in cancer development	XM002881793	18	1.5
F22	Significant	*Solanum lycopersicum* uncharacterized LOC101260735 (LOC101260735), mRNA	Uncharacterized protein	XM004241472	39	2.5
F27	Significant	*Vitis vinifera* contig VV78X019728.23, whole genome shotgun sequence	Hypothetical protein	AM446429	18	1.1
F28	Significant	*Vitis vinifera* rab GDP dissociation inhibitor alpha-like (LOC100261021), mRNA	Regulation of Rab small G proteins	XM002280570	90	2e-77
F31	Significant	*Arabidopsis lyrata* subsp. lyrata predicted protein, mRNA	Hypothetical protein	XM002877426	25	9.4
F32	Moderate	Glycine max AP-1 complex subunit mu-1-I-like	Required for many aspects	XM003551989	29	0.20

Table 1 (continued)

cDNA	Upregulation	Gene identified	Biological function	Accession number	Query age (%)	*E* value
		(LOC100789477), mRNA	of development and behavior			
F33	Moderate	*Indocalamus emeiensis* voucher Zeng and SD Zhang 07001 trnC-rpoBintergenic spacer, partial sequence; chloroplast	Plant molecular systematic studies	GU354461	25	0.052
F38	Significant	*Ricinus communis* 60S ribosomal protein L14, putative, mRNA	Protein synthesis	XP002529840	63	3e-75
F39	Significant	*Zea mays* clone 1685440 hypothetical protein mRNA, complete cds	Hypothetical protein	EU958326	32	2.1
F52	Slight	*Lotus japonicus* clone JCVI-FLLj-5P5 unknown mRNA	Unknown	BT134688	33	0.002
R1	Significant	*Gossypium hirsutum* mitogen-activated protein kinase kinase 9-like (MKK9) mRNA, complete sequence	Cellular responses to a diverse array of stimuli	HM989878	22	0.029
R8	Moderate	*Gossypium hirsutum* bacterial-induced peroxidase mRNA, complete cds	Defense against pathogen or insect attack	AF155124	42	2e-16
R16	Moderate	*Populus trichocarpa* predicted protein, mRNA	Hypothetical protein	XM002336720	22	0.086
R33	Moderate	*Arabidopsis thaliana* AtPSK1 gene for phytosulfokine precursor 1, complete cds	Regulation of defense response	AB074572	25	0.015
R38	Significant	*Populus trichocarpa* predicted protein, mRNA	Hypothetical protein	XM002300732	51	1e-28
R53	Slight	*Ricinus communis* conserved hypothetical protein, mRNA	Hypothetical protein	XM002518976	23	0.28

in cotton cultivars [31] and the correlation between increased activity of POD during discordant interactions and the use of phenolic compounds to reinforce cell walls was also reported [32].

The ribosomal protein L16 is encoded by the chloroplast gene rpl16. The amplicon F8 showed 34 % homology with *Brassica oleracea* rpl16 gene, partial sequence; chloroplast. In Arabidopsis, it has been indicated by Southern analysis that rpl16 is encoded by three genes, i.e., rpl16A, rpl16B, and rpl16C [33]. It has been observed that the protein products of rpl16A and rpl16B are not localized to ribosomes only; instead, they may perform many other functions as well. For example, rpl16A gene has an analogy with rpS6 gene which shows developmentally regulated phosphorylation [34] and it has been suggested that the protein plays a role in controlling cell growth that is a nonribosomal regulatory in nature [35]; therefore, it has been assumed that developmentally regulated expression of rpl16A serves as to increase cell proliferation rate rather than increase in ribosomes number [33].

The R33 fragment showed 25 % homology to *Arabidopsis thaliana* AtPSK1 gene for phytosulfokine precursor 1. Phytosulfokines (PSKs) belong to plant peptides class which was

discovered through growth factors that mediate density-dependent growth in cell culture study [36]. In Arabidopsis, six genes have been discovered that encode PSKs (AtPSK1-6). It has been found that 8–10 amino acids upstream from the mature peptide sequence of the propeptide precursors of PSK have conserved dibasic residues [36] and subtilases; subtilisin-like proteases are the characteristic dibasic residues of substrates sites [37]. The fragment F20 showed 18 % homology to *Arabidopsis lyrata* subsp. lyratabZIP transcription factor family protein, mRNA. bZIP (AtbZIP17) is a membrane-associated factor targeted by plant subtilisin-like serine proteases (subtilase). Our results also corroborate with the findings of Liu et al. [38] who reported that when AtbZIP17 is cleaved, the N-terminal bZIP component is translocated to nucleus and the expression of salt stress response genes are activated there.

The amplicon R1 showed 22 % homology to *Gossypium hirsutum* mitogen-activated protein kinase kinase 9-like (MKK9) mRNA. Through mitogen-activated protein (MAP), a protein phosphorylation cascade channeled downstream is initiated by SOS2 and possibly other Ca^{+2}-activated protein kinases [39]. Our finding also coordinates with Teige et al. [40] who implicated in salt stress responses a MAP kinase kinase (MKK2) and two MAP kinases (MPK4 and 6). It has been observed that a salt-elicited Ca^{+2} signal is involved in the signaling pathway sensed by SOS3 [41] and the activation of SOS2 (a serine/threonine protein kinase) [42]. The activity and the expression level of SOS1 (a plasma membrane Na^{+}/H^{+} antiporter) is regulated by SOS2 and SOS3 together [43].

Conclusion

In conclusion, in this study in response to fungal stress, a very few cDNA fragments were recognized which have defensive roles against the pathogens in *M. oleifera*. The chances of novel gene expression against stress in *M. oleifera* would be inferred due to low output of amplicons in searching homologies with known genes but to report the novelty of these genes requires further confirmatory analysis. Therefore, the identified genes from *M. oleifera* can also be set as probes to assess the fungal inductive gene expression in plants. So there is a growing need for new, environmental-friendly bioactive agents that may be used safely in medicine, industry, and agriculture to control plant pathogens and spoilage organisms postharvest.

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