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IN-SILICO IDENTIFICATION OF MICRORNA-LIKE RNAs AND THEIR REGULATING TARGET FUNCTIONS IN *Fusarium graminearum*, A HEAD BLIGHT PATHOGEN OF WHEAT

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ABSTRACT

Different types of sRNA molecules regulating gene expression during many biological processes, from growth and development to defence against biotic and abiotic stresses have been identified previously. The fungi originated sRNAs, which are homologous to plant and animal originated miRNAs, are often termed miRNA-like RNAs (milRNAs). *Fusarium graminearum* is a devastating pathogen of wheat and other small grain cereals and causes *Fusarium* Head Blight (FHB) disease. In the present study, *in-silico* tools were used to identify milRNAs and their targets regulating different functions in *F. graminearum* using the EST approach. Additionally, functional annotation, gene ontology and pathway analysis of identified target transcripts were also done. Ten milRNA families were identified. Meaningful regulations of target transcript by identified milRNAs were computationally evaluated. Our results further confirmed that uracil was the predominant base in the identified mature milRNA sequences, while adenine and uracil were predominant in pre-milRNA sequences. Thirteen potential targets were evaluated for 4 milRNA families. The majority of the identified target transcripts regulated by milRNAs showed a stress response. milRNA 5021 was also indicated for playing an important role in the amino acid metabolism and co-factor metabolism in *F. graminearum*. To the best of our knowledge, this is the first *in-silico* study describing milRNAs and their regulation in different metabolic pathways of *F. graminearum*. The findings are important for a better understanding of *F. graminearum* milRNAs and their targets. However, further research is required to determine the specific role of milRNAs.

(Key words: In-silico, microRNAs, milRNAs, *Fusarium graminearum*, FHB)

INTRODUCTION

Gene expression is regulated by endogenous small RNA (sRNA) molecules such as small interfering RNAs (siRNAs), Piwi-associated RNAs (piRNAs) and microRNAs (miRNAs) (Großhans and Filipowicz, 2008) . These sRNA molecules are usually non-coding and regulate the gene expression either at transcriptional or post-transcriptional levels (Knip *et al.*, 2014). Among sRNAs, miRNAs are 18 to 24 nucleotide long, non-coding RNAs. miRNAs regulate the post-transcriptional gene expression by binding or cleaving the complementary sequences on target messenger RNAs (mRNAs) (Liang *et al.*, 2010). In fungi, some sRNAs may be perfectly homologous to mature miRNAs in plants and animals. However, the prediction of their secondary structures is not always possible. This is due to the differences between characteristics and distribution of these fungi originated sRNAs and plant and animal originated miRNAs (Lee *et al.*, 2010). Hence, the fungi originated sRNAs, which are homologous to plant and animal originated miRNAs, are often termed miRNA-like RNAs (milRNAs). The milRNAs generate from pathways other than that of miRNA pathways and have been discovered recently in some filamentous fungi (Lee *et al.*, 2010; Zhou *et al.*, 2012a, 2012b; Kang *et al.*, 2013). Limited reports describing the role of milRNAs in gene regulation mechanisms of non-plant pathogenic filamentous fungi are available (Lee *et al.*, 2010; Zhou *et al.*, 2012b; Kang *et al.*, 2013). milRNA-mediated gene regulation mechanisms have also been speculated to exist in a plant pathogenic filamentous fungus belonging to the genus *Fusarium* (Chen *et al.*, 2014). However, no reports are available on identifying milRNAs and their regulating target functions in a plant pathogenic fungus *Fusarium graminearum.*

F. graminearum is a devastating pathogen of wheat and other small grain cereals and causes *Fusarium* Head Blight (FHB) disease (Xu *et al.*, 2005). Significant yield

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losses and mycotoxin accumulation in grain are the consequences of disease when the climatic conditions are favourable for its development (Parry *et al.*, 1995). The common mycotoxins produced by *F. graminearum* are trichothecenes and zearalenone that can affect human and animal health if they enter the food chain. Studies have been conducted to investigate the wheat-*F. graminearum* interaction and post-anthesis moisture have been reported to influence the FHB development and mycotoxin production (Kharbikar *et al.*, 2015). However, the roles of *F. graminearum* milRNAs in this interaction have not been investigated. With concern to mycotoxin production, a deep understanding of their regulatory system governed by the milRNAs is required to study this economically important pathogen.

Various approaches such as genetic screening, direct cloning or computational predictions can be used to characterize the sRNAs including milRNAs (Guleria *et al.*, 2012). Genetic screening for sRNAs is possible only when the genomic sequence data is available (Zhang *et al.*, 2006). Besides, direct cloning of sRNAs is difficult due to their spatial and temporal expression patterns (Zhang *et al.*, 2006). Both genetic screening and direct cloning require wet lab procedures; therefore, are expensive and time-consuming. Computational characterization of sRNAs, in contrast, involves the prediction from expressed sequence tags (EST) or genomic survey sequences (GSS). This is the cost-effective and rapid approach towards the identification of sRNAs (Guleria *et al.*, 2012). Since milRNAs are the types of sRNAs computational approach could also be utilized to characterize the milRNAs in plant pathogenic fungi such as *F. graminearum*. *F. graminearum* EST sequences available in the NCBI database were used to identify milRNAs and their target in this study. ESTs have also been used for miRNA identification in several previous studies (Zhang *et al.*, 2005; Unver *et al.*, 2010; Barozai *et al.*, 2012; Catalano *et al.*, 2012). Comprehensive milRNA target analysis is still limited due to the limited ability of the available bio-informatics tools in predicting translational inhibition and integrating transcriptome data. To overcome this problem, different types of tools at each step of this study were used to identify possible milRNA mediated regulation of wheat-*F. graminearum* interaction.

MATERIALS AND METHODS

Data retrieval and software employed

A set of reported 23,260 ESTs of *F. graminearum* were downloaded from the NCBI (www.ncbi.nlm.nih.gov/nucest) in February 2019. To remove the redundancy from the EST sequences CAP3 (Huang and Madan, 1999) was used. milRNAs and their target prediction were performed by using a comprehensive tool C-mii version 1.11 (Numnark *et al.*, 2012) from 3436 contigs. Nine sequences were excluded according to the criteria of the miRNA identification module (length exceeding limit 3000 bps) of C-mii. Online web servers psRNATarget (Dai and Zhao, 2011) and Target-align (Xie and Zhang, 2010) were used to evaluate the prediction results of C-mii. Blast2GO (Conesa and Gotz, 2008) was used for annotation of identified target transcripts.

miRNAs and their target prediction

The miRNA identification module of C-mii used in this study uses a homology search-based approach. Primary miRNA folding and precursor miRNA folding, sub-modules were used with their default parameters. To process the predicted results, the stability of the secondary structure of the pre-miRNAs needs to be evaluated. A contig was considered as a milRNA candidate if it fits in the following criteria; 1) length of predicted milRNA should be in the range of 18–24 nucleotides; 2) more than three substitutions were not allowed for the predicted mature milRNAs against the known miRNAs; 3) one arm localization of the mature milRNA within a stem-loop structure; 4) maximum 6 mismatches were allowed between milRNA sequence and milRNA* sequence of stem-loop structure; 5) A + U content should be high compared to G + C content, and 6) high negative minimal folding free energy (MFE) and high MFE index (MFEI) value of the secondary structure. Predicted milRNAs were then used for target search against all contig sequences of *F. graminearum*. The target identification module of Target-align is based on perfect or near-perfect complementarities of miRNA and its target. Target scanning was performed to search complementary sites of predicted milRNAs for all contigs. The following criteria were set for the prediction of milRNA-target genes: 1) maximum three mismatches between predicted milRNAs and target gene; 2) no mismatches were allowed for 10^{th} and 11^{th} positions of the complementary site; 3) MFE of milRNA and target duplex should be negative, and 4) no more than 4 GU pair was allowed in the complimentary alignment.

Validation

The least number of allowed mismatches for the alignment of putative milRNAs and known miRNAs is the foremost feature to get accurate results. In-silico validation was done only for milRNAs having targets in predicted results. User-submitted small RNAs and user-submitted transcript section of psRNA Target were used in this study. The same alignment approach was also used by Target-align. During validation of predicted result by Target-align 3 mismatches were allowed from base 1 to 9. The following criteria were taken into account for the validation of milRNAs and their targets. 1) Only 3 non-consecutive or consecutive mismatches were allowed; all aligned pairs having more than 3 mismatches by validating tools were filtered; 2) The sequences having aligned section were considered as final results; any predicted milRNA or transcripts showing no alignment by both tools were ignored; 3) to improve the accuracy of the results only cleavage type of inhibition was considered by psRNATarget; and 4) significance of the predicted results at $p = 0.05$ was considered for the study. Evaluated target transcripts were selected for functional annotation. BlastX was performed against the plant/

Arabidopsis thaliana protein database (PDB). Gene ontology (GO) and pathway analyses were performed with its analyzed default parameters, which also evaluates annotated results.

RESULTS AND DISCUSSION

1. milRNA prediction

After carefully considering the homology and secondary structure prediction results, 10 sequences were selected as milRNA candidates. These predicted sequences belonged to 10 different families (Table 1). Evolutionarily highly conserved milRNA families such as milR2325c and milR5549-3p were identified in this study (Fig. 1). The distribution of identified milRNAs in the investigation was not uniform. All the milRNA families identified had originated from 41 different EST transcripts belonging to distantly related organisms (Fig. 2). Given that many miRNAs are highly conserved between organisms and species (Pasquinelli *et al.*, 2000) it is likely, that a milRNA discovered in *F. graminearum* will also be present in *A. lyrate, A. thaliana, B. distachyon, B. taurus, C. elegance, D. melanogaster* or other organusms and species. Further, fifty per cent of the milRNA families identified had more than one member. For example, in the case of milR5549-3p, nineteen members were identified from the same transcript (Table 1); however, only one milRNA candidate was considered for the study.

Table 1. MicroRNA like RNAs identified in *Fusarium graminerum* Expressed sequence tags (ESTs)

Micro RNA Names	Length of miRNAs	GenBank accessions of F. graminearum ESTs	Proposed names for homologous milRNAs	Length of milRNAs	Nucleotides mismatched	Nucleotide differences	Percent identities	Matching positions		Orientations	Expect (E) Value	Scores	Characteristics	Significance levels
								From	To					
aly-miR169m-3p	19	gb|CD461226.2|	Fgr-milR169m-3p	19	3	0	100	768	752	3' - 5' Negative	0.008	34.2	Unconnserved	**
ath-miR414	21	gb|BU064970.1|	Fgr-milR414	21	2	0	100	10	29	5' - 3' Positive	2.00E-04	40.1	Unconnserved	**
ath-miR5021	20	gb|CN813513.1|	Fgr-milR5021	20	2	0	100	195	213	5' - 3' Positive	6.00E-04	38.2	Conserved	**
ath-miR5021	20	gb|CN813864.1|	Fgr-milR5021	20	2	0	100	194	212	5' - 3' Positive	6.00E-04	38.2	Conserved	**
ath-miR5021	20	gb|CN814045.1|	Fgr-milR5021	20	2	0	100	198	216	5' - 3' Positive	6.00E-04	38.2	Conserved	**
ath-miR5658	21	gb|CN811991.1|	Fgr-milR5658	21	1	0	100	225	245	5' - 3' Positive	4.00E-05	42.1	Conserved	**
ath-miR5658	21	gb|CN812019.1|	Fgr-milR5658	21	1	0	100	96	116	5' - 3' Positive	7.00E-04	38.2	Conserved	**
ath-miR5658	21	gb|CN812003.1|	Fgr-milR5658	21	2	0	100	61	80	5' - 3' Positive	2.00E-04	40.1	Conserved	**
bdi-miR9482	21	gb|BU062570.1|	Fgr-milR9482	20	3	1	95	124	141	5' - 3' Positive	0.003	36.2	Unconnserved	*

bta-miR-1814c	20	gb|DN156556.1|	Fgr-milR-1814c	20	3	0	100	80	63	3'-5' Negative	0.002	36.2	Unconserved	**
bta-miR-2325c	20	gb|BU061243.1|	Fgr-milR-2325c	19	3	1	95	158	142	3'-5' Negative	0.009	34.2	Highly conserved	*
bta-miR-2325c	20	gb|BU064103.1|	Fgr-milR-2325c	19	3	1	95	221	205	3'-5' Negative	0.009	34.2	Highly conserved	*
bta-miR-2325c	20	gb|BU064781.1|	Fgr-milR-2325c	19	3	1	95	615	631	5'-3' Positive	0.009	34.2	Highly conserved	*
bta-miR-2325c	20	gb|BU067514.1|	Fgr-milR-2325c	19	3	1	95	75	59	3'-5' Negative	0.009	34.2	Highly conserved	*
bta-miR-2325c	20	gb|BU067652.1|	Fgr-milR-2325c	19	3	1	95	39	55	5'-3' Positive	0.009	34.2	Highly conserved	*
bta-miR-2325c	20	gb|BU068177.1|	Fgr-milR-2325c	19	3	1	95	39	55	5'-3' Positive	0.009	34.2	Highly conserved	*
bta-miR-2325c	20	gb|BU068628.1|	Fgr-milR-2325c	19	3	1	95	9	25	5'-3' Positive	0.009	34.2	Highly conserved	*
bta-miR-2325c	20	gb|BU068628.1|	Fgr-milR-2325c	19	3	1	95	9	25	5'-3' Positive	0.009	34.2	Highly conserved	*
bta-miR-2325c	20	gb|BI750170.1|	Fgr-milR-2325c	19	3	1	95	615	631	5'-3' Positive	0.009	34.2	Highly conserved	*
bta-miR-2325c	21	gb|CD460403.2|	Fgr-milR-2325c	19	3	1	95	652	636	3'-5' Negative	0.009	34.2	Highly conserved	*
cel-miR-5549-3	21	gb|BU063095.1|	Fgr-milR-5549-3	21	3	0	100	523	505	3'-5' Negative	7.00E-04	38.2	Unconserved	**
cel-miR-5549-3p	21	gb|BU060552.1|	Fgr-milR-5549-3p	21	3	0	100	505	487	3'-5' Negative	7.00E-04	38.2	Highly conserved	**
cel-miR-5549-3p	21	gb|BU060553.1|	Fgr-milR-5549-3p	21	3	0	100	404	232	5'-3' Positive	7.00E-04	38.2	Highly conserved	**
cel-miR-5549-3p	21	gb|BU060821.1|	Fgr-milR-5549-3p	21	3	0	100	646	447	3'-5' Negative	7.00E-04	38.2	Highly conserved	**
cel-miR-5549-3p	21	gb|BU060822.1|	Fgr-milR-5549-3p	21	3	0	100	505	233	5'-3' Positive	7.00E-04	38.2	Highly conserved	**
cel-miR-5549-3p	21	gb|BU061182.1|	Fgr-milR-5549-3p	21	3	0	100	288	466	5'-3' Positive	7.00E-04	38.2	Highly conserved	**
cel-miR-5549-3p	21	gb|BU061183.1|	Fgr-milR-5549-3p	21	3	0	100	355	533	5'-3' Positive	7.00E-04	38.2	Highly conserved	**
cel-miR-5549-3p	21	gb|BU063096.1|	Fgr-milR-5549-3p	21	3	0	100	283	301	5'-3' Positive	7.00E-04	38.2	Highly conserved	**

cel-miR-5549-3p	21	gb|BU064649.1|	Fgr-milR-5549-3p	21	3	0	10	48	46	3'-5' Negative	7.00E-04	38.2	Highly conserved	* *
cel-miR-5549-3p	21	gb|BU065163.1|	Fgr-milR-5549-3p	21	3	0	10	46	44	3'-5' Negative	7.00E-04	38.2	Highly conserved	* *
cel-miR-5549-3p	21	gb|BU065480.1|	Fgr-milR-5549-3p	21	3	0	10	44	43	3'-5' Negative	7.00E-04	38.2	Highly conserved	* *
cel-miR-5549-3p	21	gb|BU067608.1|	Fgr-milR-5549-3p	21	3	0	10	52	50	3'-5' Negative	7.00E-04	38.2	Highly conserved	* *
cel-miR-5549-3p	21	gb|BU068806.1|	Fgr-milR-5549-3p	21	3	0	10	52	50	3'-5' Negative	7.00E-04	38.2	Highly conserved	* *
cel-miR-5549-3p	21	gb|BU068818.1|	Fgr-milR-5549-3p	21	3	0	10	52	51	3'-5' Negative	7.00E-04	38.2	Highly conserved	* *
cel-miR-5549-3p	21	gb|BI750042.1|	Fgr-milR-5549-3p	21	3	0	10	48	46	3'-5' Negative	7.00E-04	38.2	Highly conserved	* *
cel-miR-5549-3p	21	gb|CV827808.1|	Fgr-milR-5549-3p	21	3	0	10	11	20	5'-3' Positive	7.00E-04	38.2	Highly conserved	* *
cel-miR-5549-3p	21	gb|CD456493.1|	Fgr-milR-5549-3p	21	3	0	10	46	45	3'-5' Negative	7.00E-04	38.2	Highly conserved	* *
cel-miR-5549-3p	21	gb|CD459879.2|	Fgr-milR-5549-3p	21	3	0	10	23	25	5'-3' Positive	7.00E-04	38.2	Highly conserved	* *
cel-miR-5549-3p	21	gb|CV827980.1|	Fgr-milR-5549-3p	21	3	0	10	11	20	5'-3' Positive	7.00E-04	38.2	Highly conserved	* *
cel-miR-5549-3p	21	gb|EB531022.1|	Fgr-milR-5549-3p	21	3	1	95	26	28	5'-3' Positive	0.003	36.2	Highly conserved	*
dme-miR-4968-3p	22	gb|EB530937.1|	Fgr-milR-4968-3p	22	1	0	10	93	11	5'-3' Positive	1.00E-05	44.1	Conserved	* *
dme-miR-4968-3p	22	gb|CD459711.2|	Fgr-milR-4968-3p	21	3	1	95	20	18	3'-5' Negative	8.00E-04	38.2	Conserved	*

*Significant, **Highly significant

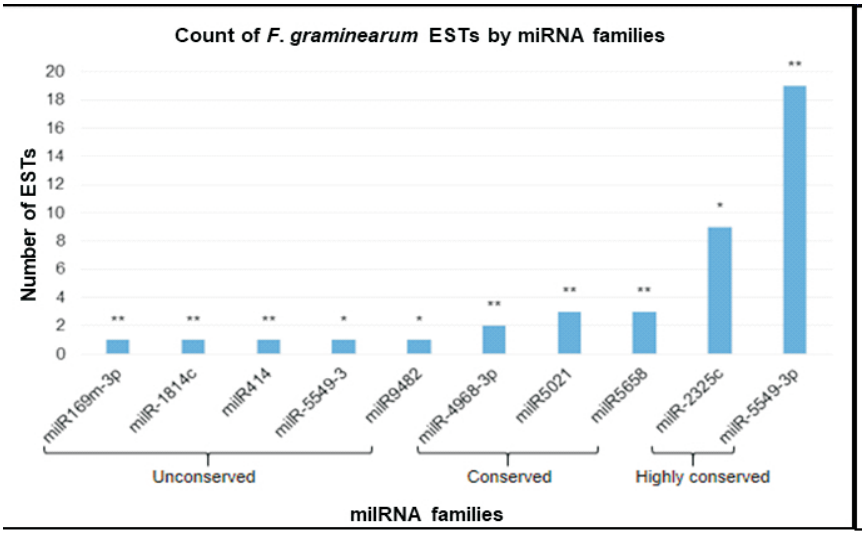

Figure 1. Graphical representation of homology of known miRNA families with 41 F. graminearum EST sequences nerating 10 milNA candidates.

Evolutionary highly conserved, conserved and unconserved milNAs were identified in the present study

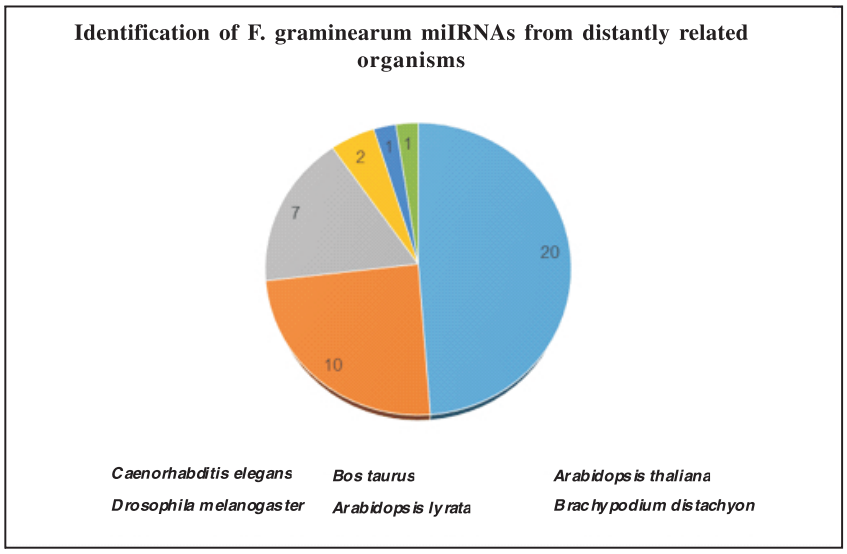

Figure 2. Distribution of *F. graminearum* milNAs in the present study. The identified milRNAs were not uniformly distributed. All the milNA families identified had originated from 41 different EST transcripts belonging to distantly related organisms

Characterization of predicted pre-milRNAs

a. Length variation

The majority of plus strands were observed for predicted milRNAs. Mature milRNA sequences showed variations from 0 to 1 nucleotide, where the 0 nt length variation was shown by 29 milRNA candidates and 1 nt length variation was shown by 12 milRNA candidates. When compared to precursor miRNAs, predicted mature milRNAs showed less variation in their length (Table 1). Variations in the length of pre-miRNAs were also reported in earlier studies (Barozai *et al.*, 2012; Wang *et al.*, 2012; Patanun *et al.*, 2013).

b. GC content

The pairing of three hydrogen bonds between G and C contributes to the formation and stabilization of the secondary structure of stem-loop hairpins. With this logic, the stability of the secondary structure of RNA should contain high GC content in the sequence. In this study, the overall range of GC varied from 30.71% to 60.14% (Table 2). The predicted milRNA families 169m-3p and 5549-3p only showed less than 40% of GC contents in their pre-milRNA sequences. Whereas, a predicted conserved milRNA family milR4968-3p and a highly conserved milR2325c showed more than 50% of GC content in their pre-milRNA sequences. Interestingly most unconserved milRNA families identified in the present study were GC rich in contrast to earlier report on *Helianthus* and *Nicotiana tabacum* (Frazier *et al.*, 2010; Barozai *et al.*, 2012). This suggest that unconserved milRNAs may also tend to have high GC content.

Compared to GC content AU content was high and varied from 35.16 to 69.28 (Table 2). Uracil was dominant in the mature milRNA sequences, suggesting its important role in milRNA mediated regulation in organisms (Zhang *et al.*, 2008; Unver *et al.*, 2010; Dhandapani *et al.*, 2011; Luo *et al.*, 2013). In most of the milRNAs, the predominance of Adenine and Uracil was observed, which is following the earlier report in *Gossypium arboretum* (L.) and *Brassica rapa* (L.), respectively (Dhandapani *et al.*, 2011; Wang *et al.*, 2012).

c. MFE and MFEI

MFE is another criterion for measuring the stability of an RNA or secondary structure. It is reported that precursor microRNAs have lower folding energies than other non-coding RNAs (Bonnet *et al.*, 2004). The MFE of the 9 predicted pre-miRNAs varied from 09.20 to 94.32 ("kcal mol^{-1}). Due to the variation in the length of precursor miRNAs, it is not enough to characterize milRNA based on MFE. The MFEI resolution for a length of variation was also calculated to distinguish miRNA from RNAs (Zhang *et al.*, 2006). The range of MFEI of predicted pre-miRNAs in *F. graminearum* varied from 0.31 to 0.81 ("kcal mol^{-1}).

1. Target prediction

Perfect or near-perfect match of miRNA to their target mRNAs helps to regulate post-transcriptional gene expression by translation inhibition and cleavage. In this study, only 4 milRNA families from the predicted milRNAs showed putative targets (Table 3). This is because the milRNA targets were highly complementary to the respective milRNAs as we focused on genes with a limited number of mismatches (Haley and Zamore, 2004). Thirteen distinct targets were predicted by Target-align. The milRNA family miR414 had 46% of all the numbers of predicted sequences for target genes. It has been reported for siRNAs, which act in a similar manner as most milRNAs, that perfect complementarity to siRNAs can be sufficient to trigger cleavage in vitro (Haley and Zamore, 2004). The majority of the identified target transcripts regulated by milRNAs showed a stress response. milRNA 5021 was also indicated for playing an important role in the amino acid metabolism and co-factor metabolism in *F. graminearum*.

Table 2. Characterization of *F. graminearum* milRNAs predicted in the present study

milRNA	PL[a]	Content					GC (%)	AU (%)	MFE (−kal mol^{-1})	MFEI (−kcal mol^{-1})	milRNA sequence
		A	U	G	C	N					
Fgr-milR1692m-3p	282	62	126	52	42	0	33.33	67.37	34.00	0.78	5′: GGCAGUCUUCUUGGCUAUC:3′
Fgr-milR-1814c	128	14	38	52	24	0	59.37	40.62	41.80	0.31	5′: GUUUUGUUUGGGUUUGUUUU:3′
Fgr-milR-2325c	182	26	38	45	73	0	64.83	35.16	54.00	0.81	5′: GGUUGUUUUUUUUCUUUUUC:3′
Fgr-milR414	119	35	24	33	27	0	50.42	49.57	23.60	0.39	5′: UCAUCUUCAUCAUCAUCGUCA:3′
Fgr-milR-4968-3p	147	32	28	46	41	0	59.18	40.82	9.20	0.34	5′: CAGCAACAGCAGCAGCAGCAGA:3′
Fgr-milR5021	148	31	54	33	29	0	42.56	57.43	31.60	0.50	5′: UGAGAAGAAGAAGAAGAAAA:3′
Fgr-milR-5549-3	349	93	110	65	81	0	41.83	58.15	81.34	0.55	5′: CUUGUGAAAUUAACGUGAGU: 3′
Fgr-milR-5549-3p	267	83	102	44	38	0	30.71	69.28	49.70	0.60	5′: UCAUGUUGGUUUUUUGUUGGU:3′
Fgr-milR5658	133	24	51	32	26	0	43.60	56.39	27.50	0.47	5′: AUGAUGAUGAUGAUGAUGAAA:3′
Fgr-milR9482	138	40	15	60	23	0	60.14	39.85	94.32	0.29	5’: CCUUUGGGGAAGAAGGGAAAC:3’

[a]Length of precursor miRNA

Table 3. Target prediction, validation, annotations and pathway analysis of the milRNA target genes using Blast2GO

milRNA	Target	Target protein	Target function	Enzymes	Pathways
Fgr-milR414	Contig 1374	High mobility group b3 protein	Chromatin assembly or dissemble/nucleic acid metabolic process	–	–
	Contig 2136	Ring u-box domain-containing protein	Protein degradation/signal transduction	–	–
	Contig 2184	Unknown	–	–	–
	Contig 277	Nascent polypeptide-associated complex subunit alpha-like protein 4	Response to stimulus	–	–
	Contig 2774	Unknown	–	–	–
	Contig 3094	Histone deacetylase hdt2	Regulation of transcription/regulation of gene expression, epigenetic	–	–
Fgr-milR169m-3p	Contig 665	3-Ketoacyl-synthase 6	Fatty acid biosynthesis/response to stress/ developmental process	–	–
Fgr-milR5021	Contig 1735	hxxxd-type acyl-transferase-like protein	Transferase activity	EC: 2.1.1.14 EC: 2.7.7.3	Cysteine and methionine metabolism/ selenocompound metabolism Pantothenate and CoA biosynthesis
	Contig 1888	Non-specific lipid-transfer protein 7	Transferase activity	EC: 2.5.1.6	Cysteine and methionine metabolism
	Contig 2208	Ubiquitin-conjugating enzyme e2 19-like	Ubiqutin-dependent protein catabolic process	–	–
Fgr-milR5658	Contig 2520	Ubiquitin-specific protease family c19-related protein	Ubiqutin-dependent protein catabolic process	–	–
	Contig 896	Peptide methionine sulfoxide reductase b2	Response to stress	–	–
	Contig 1974	Gram domain family protein	Response to biotic and abiotic stress	–	–

3. Validation

Both milRNAs and their targets were predicted by using selected criteria as mentioned earlier. Results obtained by Target-align suggested the possible role of predicted milRNAs in the down-regulation of corresponding targets. Variation between the evaluated and predicted results for milRNAs and their targets was analyzed and the result was categorized based on milRNA families (Table 3). The rest of the milRNA families had a significant match with the predicted transcript by validation. This suggests that for milR414, six predicted target transcripts were evaluated successfully. Both evaluating tools showed alignment with the same transcript. In the case of milR5021, two transcripts were validated. A small variation was observed for predicting transcript contig 1735, which was evaluated by Target-align but did not yield any results for this transcript. In this investigation, only those results were considered which showed results with at least one tool. An extra transcript contig 2528 was suggested by Target-align, for which there were no prediction results. Hence, this transcript was ignored for this family. Target-align results suggested that all evaluated results are responsible for post-transcriptional regulation by cleavage inhibition.

4. The multiplicity of a target site

miRNAs are known to have multiple target sites on a specific target transcript (Axtell *et al.*, 2006). Recognition activity of the miRNA to the mRNA target can be measured by the multiplicity of target sites. In this study, the multiplicity of the evaluated milRNA-target transcript with the difference of more than three consecutive nucleotide positions was considered as multiple target sites. All evaluated tools showed variation in the multiplicity of the target sites. According to the analyzed result, the majority of the target transcripts have more than one binding site for miRNAs. More binding sites of target transcripts are suggested by evaluating tools for identified miRNAs presenting more accuracy for miRNA-target duplex.

3. Annotation

Understating the target functions of milRNAs is highly important as it helps to understand the functional regulation of milRNAs. According to C-mii annotation contig 2184 and contig 2774 coding for transcription initiation factor IIF subunit alpha and 60 ribosomal proteins play a major role in positive regulation of transcription and translation, respectively. No function was found for contig 2184 and contig 2774 by Blast2GO. Analysis of ten sequences using Blast2GO yielded similar results as C-mii annotation. Environmental stresses are an important limiting factor for growth and development in organisms. There are reports that during stress the mycotoxin biosynthesis increases in *F. graminearum* (Edwards *et al.*, 2012; Kharbikar *et al.*, 2015). Additionally, different elicitors accumulated during stress also increase its mycotoxin biosynthesis. The results of this investigation are directly not related to mycotoxin biosynthesis but related to stress response (Table 3). Hence, the result also has importance to study the relationship of milRNA-mediated response to mycotoxin production. Additionally, the study reveals that identified milRNA regulated target genes had differential biological functions including transferase activity, fatty acid biosynthesis and signal transduction (Table 3).

4. Pathway regulation by milRNA5021

Plant enzymes obtained through Blast2GO analysis were considered for the study (Table 3). Methionine works as an active precursor of S-adenosylmethionine (AdoMet) which is a major methyl group donor in trans-methylation reaction. Moreover, being an intermediate in the biosynthesis of polyamines and the phytohormone ethylene, it plays important role in plant development. Alteration in plant morphology and induction in homeotic alteration in flower organs are responses to the methylation of cytosine residues in DNA (Finnegan *et al.*, 1998). Our study revealed that milR5021 may control the growth by regulating S-adenosylmethionine synthase (EC: 2.5.1.6) and homocysteine methyltransferase (EC: 2.1.1.14) in cysteine and methionine metabolism. Additionally, seleno compound metabolism may also be regulated by the same milRNA by targeting homocysteine methyltransferase (EC: 2.1.1.14). Apart from this amino acid metabolism, milRNA5021 may regulate the growth, stress resistance and seed lipid storage through targeting pantethine-phosphate adenyltransferase (EC: 2.7.7.3) in the pantothenate CoA biosynthetic pathway (Rubio *et al.*, 2008). A homologous miRNA, miR5021 have already been reported to play a role in oxidative stress, methyltransferase activity and terpenoid biosynthetic process in *Catharanthus roseus* (Pani and Mahapatra, 2013).

Comprehensive plant milRNA target analysis is still limited despite the availability of tools. These tools have limited ability to predict translational inhibition and integrate transcriptome data. To overcome this problem, different types of tools at each step of this study were used. The present investigation predicted 10 milRNA families in *F. graminearum* using the bioinformatics method. In addition, 13 targets could be identified for 4 milRNA families. All predicted milRNAs and targets were computationally validated. Validated milRNAs showed a response to environmental stress. With concern to pathogenicity, this study may provide a deep understanding of the regulatory system of mycotoxins governed by the milRNAs, by the relationship of milRNA-mediated response to mycotoxin production. This study presents the first report for the *in-silico* identification of *F. graminearum* milRNAs and their targets. These findings are important for a better understanding of *F. graminearum* milRNAs and their targets. Further research is required to determine the role of milRNAs in the regulation of mycotoxins by *F. graminearum*.

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