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Identification of potassium phosphite responsive miRNAs and their targets in potato

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Abstract

Micro RNAs (miRNAs) are small single strand non-coding RNAs that regulate gene expression at the post-transcriptional level, either by translational inhibition or mRNA degradation based on the extent of complementarity between the miRNA and its target mRNAs. Potato (*Solanum tuberosum* L.) is the most important horticultural crop in Argentina. Achieving an integrated control of diseases is crucial for this crop, where frequent agrochemical applications, particularly fungicides, are carried out. A promising strategy is based on promoting induced resistance through the application of environmentally friendly compounds such as phosphites, inorganic salts of phosphorous acid. The use of phosphites in disease control management has proven to be effective. Although the mechanisms underlying their effect remain unclear, we postulated that miRNAs could be involved. Therefore we performed next generation sequencing (NGS) in potato leaves treated and non treated with potassium phosphite (KPhi). We identified 25 miRNAs that were expressed differentially, 14 already annotated in miRBase and 11 mapped to the potato genome as potential new miRNAs. A prediction of miRNA targets showed genes related to pathogen resistance, transcription factors, and oxidative stress. We also analyzed *in silico* stress and phytohormone responsive cis-acting elements on differentially expressed pre miRNAs. Despite the fact that some of the differentially expressed miRNAs have been already identified, this is to our knowledge the first report identifying miRNAs responsive to a biocompatible stress resistance inducer such as potassium phosphite, in plants. Further characterization of these miRNAs and their target genes might help to elucidate the molecular mechanisms underlying KPhi-induced resistance.

Introduction

Potato (*Solanum tuberosum* L.) is the fourth food crop worldwide and the most important horticultural crop in Argentina, where 80,000 hectares of potatoes are planted per year, mainly in the provinces of Córdoba and Buenos Aires [1]. Integrated control of diseases is crucial for this crop, where frequent agrochemical applications, particularly fungicides, are carried out to manage late blight disease caused by *Phytophthora infestans* [2,3]. An alternative strategy is based on promoting induced resistance through the application of environmentally friendly

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compounds. In this context, phosphites have been extensively used as biocompatible inducers of defense responses [4].

The role of phosphites in disease control management has been studied extensively, and previous results have shown many promising properties associated with these compounds: the stimulation of plant defense mechanisms such as enhanced production of reactive oxygen species (ROS), the induction of pathogenesis related proteins (PRs) and the reinforcement of the cell wall [5–7]. It has also been demonstrated that KPhi primes an intense and rapid response to infection, involving heightened activation of a range of defense responses [8–10]. Additionally, KPhi has been proven to be an effective protective agent against UV-B stress [11]. However the mechanisms underlying KPhi protective effect remain unclear, the complexity of defense mechanisms and the plethora of pathways involved, suggest that miRNAs might be involved. miRNAs can target and subsequently regulate the expression of multiple genes simultaneously, providing a rapid and efficient way of regulating plant responses to stress.

miRNAs are small single-stranded, non-coding RNAs present both in animals and plants, that regulate gene expression at the post-transcriptional level, either by repressing mRNA translation or mediating the degradation of the targeted mRNAs depending on their degree of complementarity [12]. Specifically in plants, this class of ~22 nt RNAs play crucial roles in plant biological processes and responses to disease and environmental stresses [13–15]. Many plant miRNAs and their targets have been identified by computational and experimental approaches, in various species [16,17]. Several conserved miRNA families have been described in potato, some of them targeting transcription factors with diverse roles in plant growth and development, genes involved in signal transduction, and hormone signaling pathways. miRNA families such as mir172, miR156, miR164, miR166, miR167, miR171, miR390, miR394, miR395, miR399, miR530, miR829, mir395, mir398, miR414, miR778, among others, appear to be involved in potato response to various disease and environmental stresses [18–20].

We have hypothesized that one or more miRNAs might control the key-steps that lead to defense reactions mediated by KPhi in potato. In this scenario, the present work aims to identify miRNAs involved in the regulation of potato defense responses after potassium phosphite treatment.

Materials and methods

Plant material and phosphite treatment

S. tuberosum seed tubers (cv. Shepody) were planted in pots containing a pasteurized mixture of soil and vermiculite (3:1, v/v). Pots were maintained under greenhouse conditions (18˚C day-night temperature, 16 h of light per day). Potassium phosphite (KPhi), 1% (v/v) water solution of the commercial product (Afital Potassium Phosphite, Agro-EMCODI SA) was applied to the foliage at 5 mL per plant (3 L/ha) by using an atomizer (ESAC SA) operating at 200 kPa, 21 days after emergence. Control plants were sprayed with distilled water. Leaf tissue was collected after 72 hs of KPhi or water treatment. Experiments were performed at least three times for each condition.

RNA isolation

Total RNA from each treatment was isolated from 0.1 g of fresh leaf tissue using the RNeasy kit (Qiagen), following the manufacturer's protocol. The purified RNAs were analyzed using a Thermo Scientific NanoDropOne spectrophotometer. RNA integrity was checked by 1% agarose gel electrophoresis. Three biological replicates were performed for each condition: control (C) and KPhi treated (T).

Next generation sequencing

Total RNA (1 ug) was used to prepare small RNA libraries according to the TruSeq Small RNA Sample Prep Kits protocol (Illumina, San Diego, USA). Purified cDNA libraries were 50bp single-end sequenced on an Illumina Hiseq 2500 at LC Sciences (Houston, Texas, USA) following the vendor's recommended protocol.

Identification of known and novel miRNAs

Raw reads were subjected to an in-house program, ACGT101-miR (LC Sciences, Houston, Texas, USA) to remove adapter dimers, contaminating sequences with no 3' adapters (3ADT), low complexity regions, common RNA families (rRNA, tRNA, snRNA, snoRNA) and repeats. Subsequently, unique sequences with 18~25 nucleotides length were mapped to *Solanum tuberosum* (specific species) precursors in miRBase 21.0 by BLAST search to identify known miRNAs and novel 3p- and 5p- derived miRNAs. Length variation at both 3' and 5' ends and one mismatch inside of the sequence were allowed in the alignment. The unique sequences mapping to specific species mature miRNAs in hairpin arms were identified as known miRNAs. The unique sequences mapping to the other arm of known specific species precursor hairpin opposite to the annotated mature miRNA-containing arm were considered to be novel 5p- or 3p derived miRNA candidates. The remaining sequences were mapped to *Solanum lycopersicum* precursors (selected species of *Solanaceae* family) in miRBase 21.0 by BLAST search, and the mapped pre-miRNAs were further BLASTed against the specific species genomes to determine their genomic locations. The above two were defined as known miRNAs. The unmapped sequences were BLASTed against the specific genomes, and the hairpin RNA structures containing sequences were predicted from the flank 120 nt sequences using RNAfold software (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). According to the mapping performed and secondary structure prediction reads were assigned to different groups (as described in detail in Fig 1).

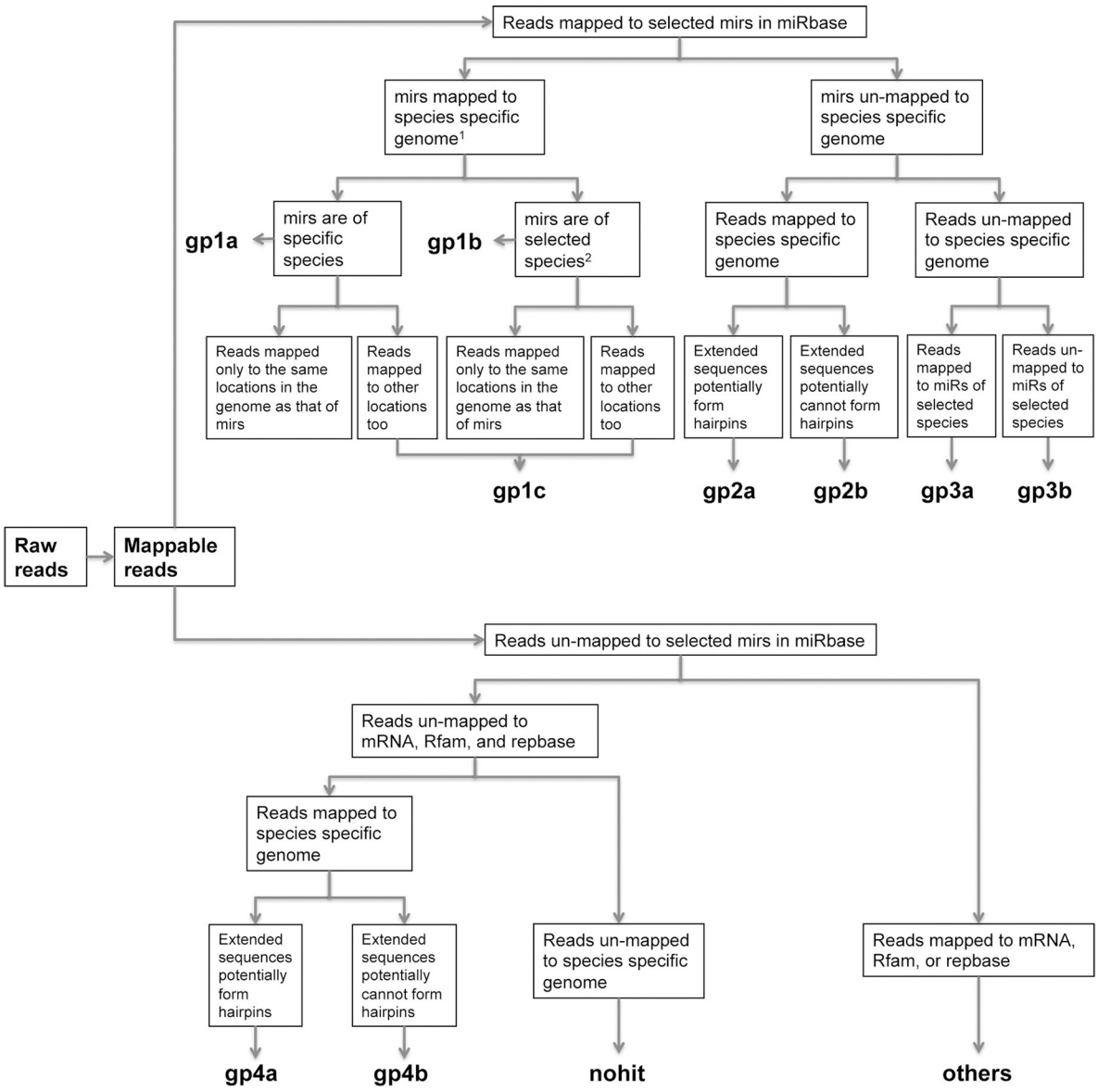
The naming system of miRNAs identified in this study is as follows: the miRNA name is composed of the first known miR name in a cluster, an underscore, and a matching annotation: L–n means the miRNA_seq (detected) is n base less than known rep_miRSeq in the left side; R–n means the miRNA_seq (detected) is n base less than known rep_miRSeq in the right side; L+n means the miRNA_seq (detected) is n base more than known rep_miRSeq in the left side; R+n means the miRNA_seq (detected) is n base more than known rep_miRSeq in the right side; 1ss8GA means 1 substitution (ss), which is G to A at position 8. If there is no matching annotation, the miRNA_seq (detected) is exactly the same as known rep_miRSEq. miRNAs located on the other arms of hairpin structures are annotated as p5/p3 to distinguish from the reported 5/3 sequences.

Differential expression analysis

Differential expression of miRNAs based on normalized deep-sequencing counts was analyzed using Student's t-test. The significance threshold was set to 0.01 and 0.05 in each test. Sequence reads of the six libraries were normalized to 1 million by the total number of clean small RNA reads in each sample. The $\log_2$ ratio formula was as follows: $\log_2$ ratio = $\log_2$ (miRNA reads in KPhi treated/miRNA reads in control).

Target prediction and GO analysis

Target predictions were performed with psRNATarget web server (http://plantgrn.noble.org/psRNATarget/analysis) using default parameters with a maximum of 3 expectation cut-off [21]. To better understand their function, the putative target genes of the differentially



[1] *Solanum tuberosum*
[2] Solanaceae

Fig 1. Flow chart of data analysis.

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expressed miRNAs were subjected to GO analysis. Significant GO terms were calculated by a Hypergeometric equation, and those GO terms with p-value<0.05 were defined as significant.

qRT-PCR analysis of miRNAs and their predicted targets

The expression level of miRNAs was detected by stem-loop based reverse transcription and quantitative real time PCR (qRT-PCR) [22]. miRNAs and mRNAs were reverse transcribed to

cDNAs with stem-loop specific primers and oligo dT primers, respectively, using the GoScript (TM) Reverse Transcriptase (Promega). cDNA was generated from 1ug of total RNA, with both miRNA specific stem-loop primers and oligo dT primers in each reaction tube at 16˚C for 30 minutes, 42˚C for 30 minutes, 50˚C for 60 minutes and 70˚C for 15 minutes.

The expression levels of miRNAs and mRNAs were analyzed by quantitative real-time PCR (qPCR) using an Applied Biosystems StepOneTM Plus Real-Time PCR System (Applied Biosystems, Waltham, MA, United States) and FastStart Universal SYBR Green Master mix (Roche). For miRNAs, samples were incubated at 95˚C for 5 minutes, and the amplification was set at 45 cycles of denaturation at 95˚C for 15 seconds, annealing at 60˚C for 30 seconds, and an extension step at 72˚C for 20 seconds. For miRNA target genes, samples were incubated at 95˚C for 10 minutes, and amplification was set at 40 cycles of denaturation at 95˚C for 15 seconds and an annealing step at 60˚C for 1 minute. miRNA and target-gene specific primers were designed using NGS data and available databases (http://www.mirbase.org/, http://solgenomics.net/) (S1 Table). *EF1α* was employed as an internal control for mRNAs and miRNAs. Reactions were performed with three biological replicates and relative gene expression level was analyzed using the comparative $2^{\Delta\Delta}$CT method [23]. Pair-wise fixed reallocation randomization test statistical analysis was performed with REST software (Relative Expression Software Tool) [24].

Analysis of cis-acting elements

The presence of well characterized cis-acting elements in the promoters of pre-miRNAs was analyzed *in silico* using the web available software PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/search_CARE.html) [25]. Upstream sequences of potato pre-miRNAs (1000 bp) were retrieved from the PGSC v4.03 database (http://solanaceae.plantbiology.msu.edu/).

Availability of supporting data

The data supporting this work are available in the public database: Gene Expression Omnibus (GEO), under the accession number GSE132232.

Results

High throughput sequencing of small RNAs

Total RNA from potassium phosphite treated (T2, T4, T6) and non treated leaves (C1, C3, C5) was isolated, and six small RNA libraries were constructed to perform high throughput sequencing. RNAseq from the libraries generated 58,391,355 total raw reads. In particular, C1 library yielded 8,599,726 reads, while C3 and C5 libraries yielded 8,051,010 and 8,175,531 reads, respectively. T2, T4, and T6 yielded 12,864,315, 10,563,970 and 10,136,803 reads, respectively (S2 Table).

After low-quality reads, adapters, poly A sequences and short RNAs shorter than 15 nucleotides were removed, 32,677,778 unique small RNA reads (56% of the total raw reads) remained (Fig 2A). RNA length ranged between 15–32 nt, with the highest proportion corresponding to 21 nt (Fig 2B). Reliable reads were divided into 4 groups as described in Fig 1. A total of 206 *S. tuberosum* known miRNAs (group 1a) were identified along with additional 42 *Solanaceae* miRNAs (group 1b), novel to potato. Interestingly, 388 reads that mapped to the potato genome, presented extended sequences that potentially form hairpins constituting potential novel miRNAs (group 4a) (Table 1).

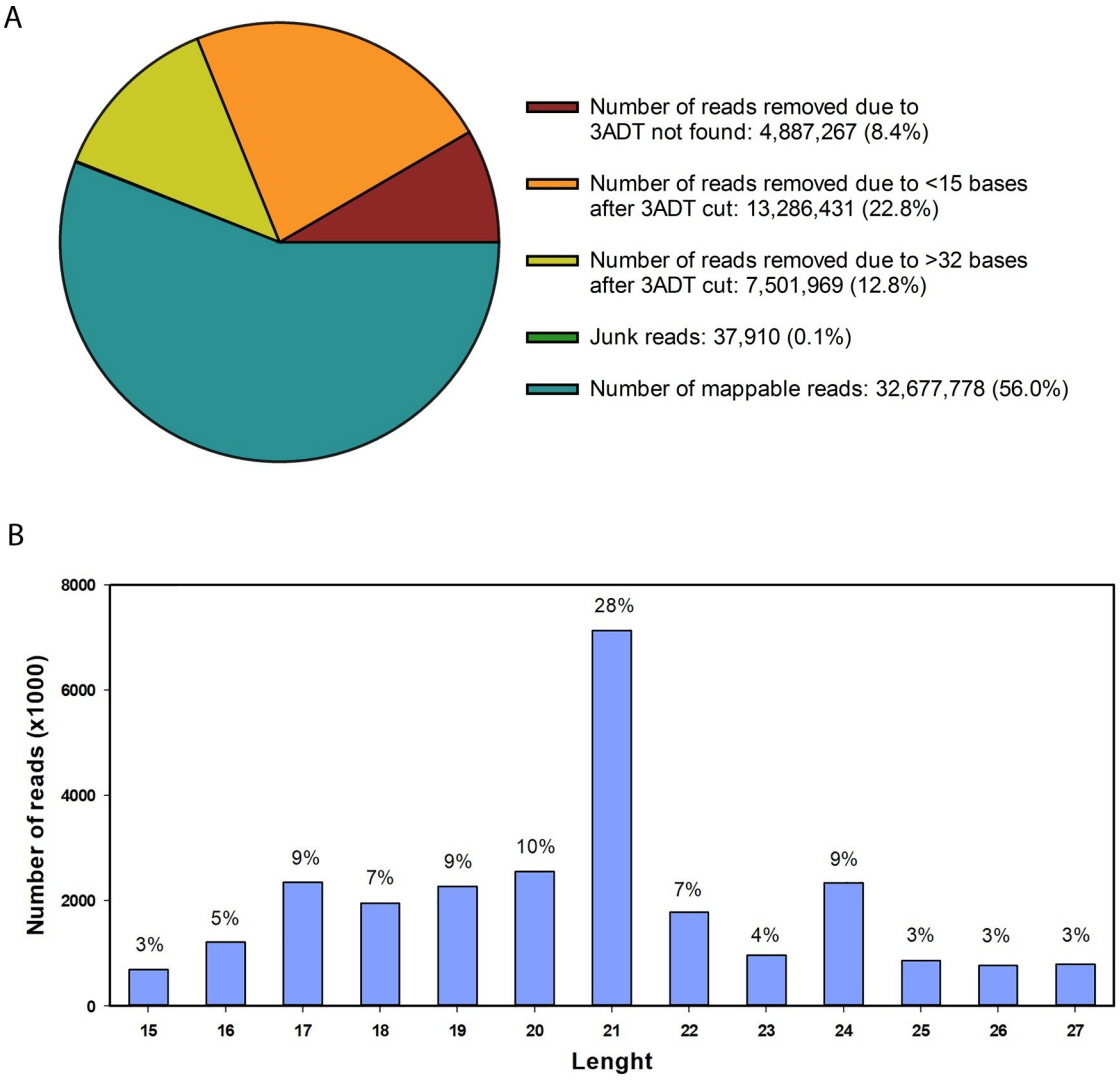

Fig 2. Data filtering and length distribution of mappable reads. Raw reads were filtered to remove sequences with no 3' adapters (3ADT), junk reads and reads shorter than 15 and longer than 32 nucleotides; and were subsequently mapped to specific and selected genomes. (A) Pie plot of data filtering. (B) Length distribution of mappable reads.

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Differential expression analysis of known and novel miRNAs after KPhi treatment

To identify differentially expressed miRNAs after KPhi treatment, six small RNA libraries were constructed from treated and non treated plants and sequenced independently. Statistical analysis showed that 25 miRNAs were differentially expressed after KPhi treatment (Fig 3, S3 Table). Among them, 14 were already annotated in the miRBase, and 11 were mapped to the potato genome as potential novel miRNAs (PCs). From these 14 known miRNAs, 3 were up-regulated (stu-miR398b-3p, sly-miR167b-5p and stu-miR4376-5p L-1R+2) and 11 were down-regulated (stu-miR166b, stu-miR482c, stu-miR482a-3p, sly-miR159-p5, stu-miR171a-5p, stu-MIR7985-p5, sly-miR166c-3p_R+1, sly-miR166a_R+1, sly-miR171d_R+1_1ss8GA, stu-miR530_L-2R+2, sly-MIR166b-p5).

Among the 11 potentially new miRNAs, 8 were up regulated (PC-3p-48036_37, PC-3p-26055_79, PC-5p-1305_1307, PC-3p-2979_661, PC-3p-6013_346, PC-3p-40282_47, PC-5p-

Table 1. Summary of data analysis results.

		Group	# of sequences	# of unique miRNAs
	Raw		58,391,355	
	Total mappable reads		32,677,778	
Known miRNAs	of specific species[1]	1a	3,506,752	206
	of selected species[2], but novel to specific species Group	1b	113,297	42
Predicted miRNAs	Mapped to known miRNAs of selected species and genome; within hairpins	2a	1,515,585	38
	Mapped to known miRNAs of selected species and genome; no hairpins	2b	871	132
	Mapped to known miRNAs and miRNAs of selected species but unmapped to genome	3a	1,230	52
	Mapped to known miRNAs of selected species but unmapped to genome	3b	550	52
	Unmapped to known miRNAs but mapped to genome and within hairpins	4a	185,614	388
	Overall			910
	Others (mapped to mRNAs, RFam, or repbase)		7,554,022	
	Nohit		7,916,889	

[1] *Solanum tuberosum*

[2] *Solanaceae*

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14130_152, PC-5p-4138_490) and 3 were down regulated (PC-5p-30841_65, PC-5p-10686_200, PC-3p-14680_147).

To confirm the high-throughput sequencing data, stem loop qRT-PCR was performed for 7 of the differentially expressed miRNAs. A comparison of the results from qPCR with those from NGS revealed similar patterns of expression (Fig 4).

Target prediction and GO analysis

To elucidate the biological functions of KPhi differentially expressed miRNAs, mRNA sequence complementarity was assessed using the psRNATarget software. A total of 211 potential target genes were identified for the 14 conserved miRNAs, based on their perfect or near-perfect complementarity to their target mRNA sequences. For most of the differentially expressed miRNAs, more than one potential target gene was predicted. Additionally, several target genes (a total of 118) were identified for most of the potentially novel miRNAs. Most of the predicted targets belong to transcription factor gene families, such as bZip, GRAS, AP2, REV HD-ZipIII, ARF, among others. Other miRNAs were predicted to target genes involved in the regulation of plant metabolism and stress responses. Detailed annotations of these results are presented in S4 Table.

Some of the miRNA predicted targets were analyzed by qPCR (Fig 4, Table 2). These results showed that 6 of the analyzed targets followed a negatively correlated expression pattern with their miRNAs. However, miRNA171a-5p target (methylketone synthase Ib) expression, was not consistent with its miRNA down-regulation (Fig 4).

To further analyze the biological function of miRNA targets, GO analysis was performed. The highest percentage of genes falls into defense response, regulation of transcription, and signal transduction categories (biological process group). Cellular components and molecular functions of most of the genes are consistent with their biological process group (Fig 5, S5 Table)

In-silico analysis of cis-elements present in KPhi responsive miRNA promoters

Several stress responsive cis-elements were identified *in-silico* in promoters of potassium phosphite differentially expressed miRNAs (Fig 6): anaerobic response element (ARE), element

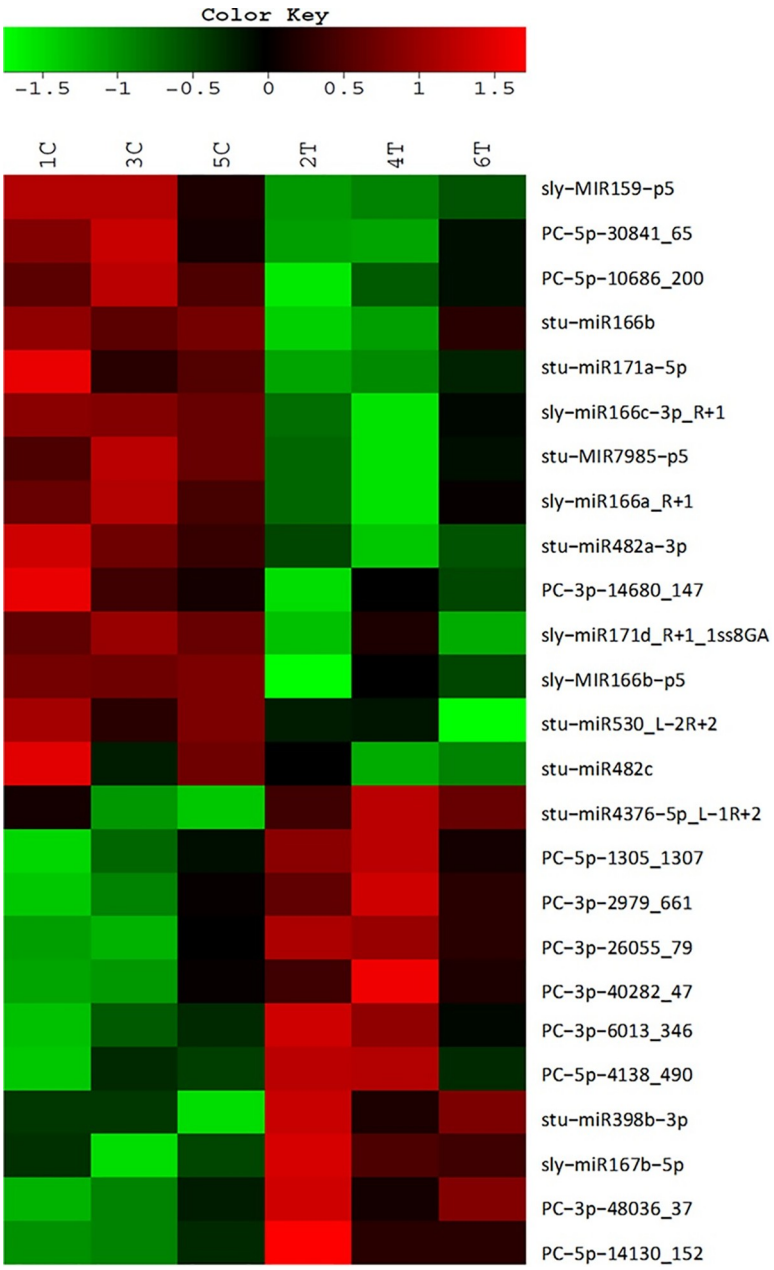

Fig 3. Heat map of differentially expressed miRNAs. KPhi treated (T2, T4, T6) and non treated (C1, C3, C5) replicates were analyzed. Differential expression of miRNAs based on normalized deep-sequencing counts, was analyzed using a Student's t-test. Up-regulated and down-regulated miRNAs are represented in red and green, respectively.

https://doi.org/10.1371/journal.pone.0222346.g003

sensitive to the fungal inducer (Box-W1), MYB binding site related to the abiotic stress (MBS) and its stress-related binding site MYBHv1 (CCAAT-box), MYB binding site involved in the regulation of flavonoid biosynthetic genes (MBSII), enhancer element involved in specific anoxic inducibility (GC-motif), cis-acting element involved in heat stress response (HSE), low temperature response element (LTR), repetitions rich in TC involved in defense and response to stress (TC-reach repeats), wound-sensitive element (WUN), UV light stress related cis-elements (box I and box G). All miRNAs presented more than one stress related cis-elements in

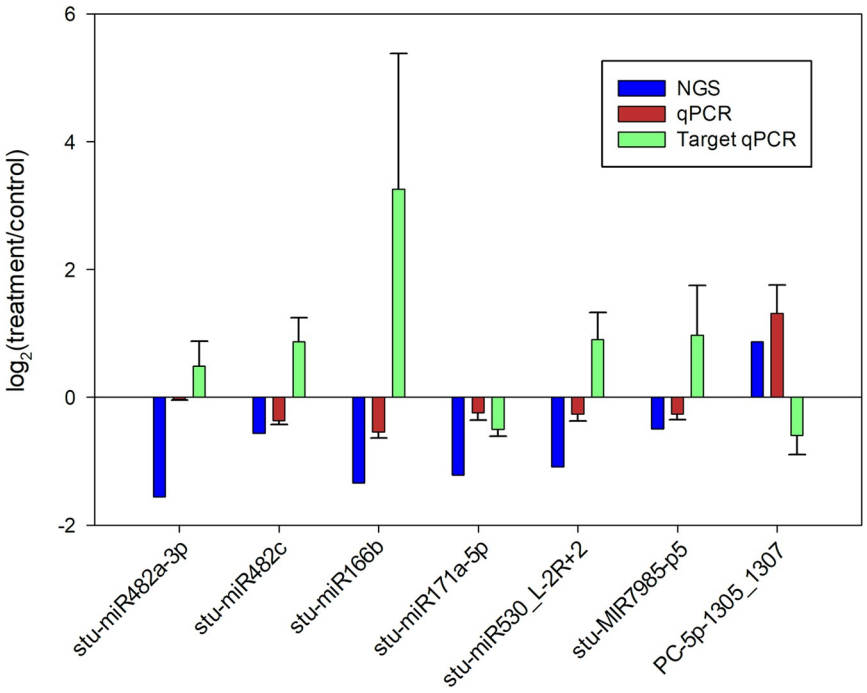

Fig 4. Validation of next generation sequencing (NGS) results for selected differentially expressed miRNAs and analysis of selected target genes. Quantitative real time PCR (qPCR) was performed to validate differentially expressed miRNAs (stu-miR482a-3p, stu-miR482c, stu-miR166b, stu-miR171a-5p, stu-miR530_L-2R+2, stu-MIR7985-p5, PC-5p-1305_1307) and targets (TIR-NBS-LRR resistance protein, Resistance protein PSH-RGH7, BZIP domain class transcription factor, Methylketone synthase Ib, Zinc knuckle (CCHC-type) family protein, Spotted leaf protein, F-box protein family). The EF1α gene was used as housekeeping. Three biological replicates were employed. Vertical bars represent the standard deviation of the mean (n = 3).

https://doi.org/10.1371/journal.pone.0222346.g004

their promoter regions, suggesting they might participate in multiple stress response signaling pathways, and some of them were in multiple copy numbers. In addition to stress-related elements, most of the KPhi differentially expressed miRNAs presented motifs associated to phytohormones: element sensitive to abscisic acid (ABRE), cis-acting regulatory element involved in the methyl jasmonic acid response (TGACG-motif), ethylene sensitive element (ERE), cis-acting element involved in the response to salicylic acid (TCA element), auxin-sensitive element (TGA element), cis-acting elements involved gibberellin response (GARE motif, P-box and TATC box).

Table 2. Selected differentially expressed miRNAs after KPhi treatment and targets.

miRNA	Differential expression under KPhi treatment	Target accession	Target description
stu-miR482a-3p	Down-regulation	PGSC0003DMT400053047	TIR-NBS-LRR resistance protein
stu-miR482c	Down-regulation	PGSC0003DMT400012486	Resistance protein PSH-RGH7
stu-miR166b	Down-regulation	PGSC0003DMT400074934	BZIP domain class transcription factor
stu-miR171a-5p	Down-regulation	PGSC0003DMT400066685	Methylketone synthase Ib
stu-miR530_L-2R+2	Down-regulation	PGSC0003DMT400002883	Zinc knuckle (CCHC-type) family protein
stu-MIR7985-p5	Down-regulation	PGSC0003DMT400062931	Spotted leaf protein
PC-5p-1305_1307	Up-regulation	PGSC0003DMT400027717	F-box family protein

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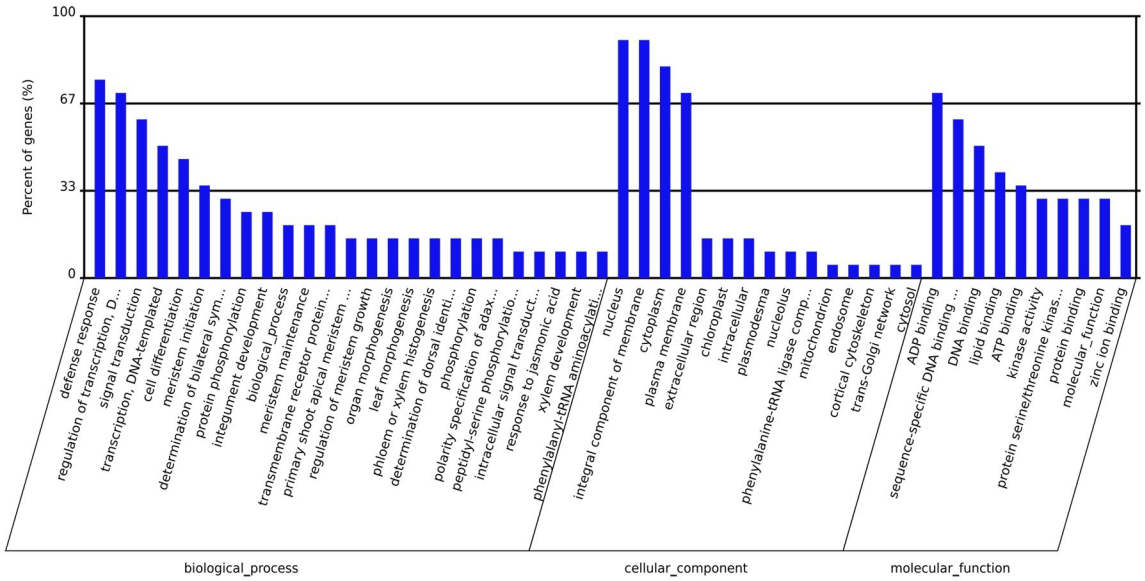

Fig 5. Gene ontology analysis of differentially expressed miRNA targets. All identified target genes were classified according to their Biological process (BP), Molecular function (MF), and Cellular component (CC) based on GO enrichment analysis. Statistical significance was set to $p<0.05$.

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Discussion

Despite the available information about the stress protective effect of KPhi on different crops, the mode of action is unclear and the target/effector molecules in the plant are still unknown.

	Phytohormone responsive								Abiotic and biotic stress responsive										
	ABRE	TGACG motif	ERE	TCA element	TGA element	GARE motif	P-box	TATC box	ARE	Box-W1	GC motif	HSE	MBS	MBSII	CCAAT box	LTR	TC-rich repeats	WUN motif	I-box	G-box
stu-miR482c				1	1			1		1		2					1	1	3	
stu-miR166b		1							1		1		1		1	1	1			2
stu-miR482a				1					2			1		1		1	1			
sly-miR159			1			1	1			1									1	
stu-miR171a	1		1			3			1			1	1	1						1
stu-miR530	5			1					1						1	1	1		2	6
sly-miR171d				1		1												1	1	
stu-miR7985		1		1		1			2				4						4	2
sly-miR166c		1		1													1		1	1
sly-miR166a		1		1					1			2	3			1	1			2
sly-miR166b		1				2	1	1		1			2						3	
stu-miR391										1	1		1			1				
sly-miR167b			1						2								2			
stu-miR398b				2					2	1	1	1	2				1			

Fig 6. *In silico* identification of cis-elements in the promoters of KPhi differentially expressed miRNAs. The promoters of pre-miRNAs were analyzed *in silico* using the web available program PlantCARE (bioinformatics.psb.ugent.be/webtools/plantcare/html). Down-regulated and up-regulated miRNAs are shown in black and grey, respectively. The numbers represent total well characterized cis-acting elements per miRNA.

https://doi.org/10.1371/journal.pone.0222346.g006

The present study aimed to gain an insight into the complex mechanisms of action of KPhi. To achieve this objective, we performed a sequencing analysis to determine changes in miRNA expression and analyzed their targets in potato leaves treated with KPhi. Additionally, to identify common cis-elements involved in miRNA regulation, we analyzed their promoters *in-silico*.

High-throughput sequencing results revealed the presence of 206 unique miRNAs belonging to potato, and 42 unique miRNAs from tomato, but not previously described in potato. Additionally, a total of 288 potential new miRNAs were identified. Differential expression analysis showed changes in the relative abundance of 25 miRNAs after KPhi treatment, 14 of them belonging to known *Solanaceae* miRNA families and 11 of them being potential novel miRNAs. qPCR results were consistent with NGS differential expression analysis for 7 miRNAs (stu-miR482a-3p, stu-miR482c, stu-miR166b, stu-miR171a-5p, stu-miR530_L-2R+2, stu-MIR7985-p5, PC-5p-1305_1307).

The analysis of putative target genes of miRNAs responsive to KPhi treatment gave an insight into the potential mechanisms and effectors of this compound. In this context, we analyzed the expression of 7 target genes: TIR-NBS-LRR resistance protein, Resistance protein PSH-RGH7, BZIP domain class transcription factor, Methylketone synthase Ib, Zinc knuckle (CCHC-type) family protein, Spotted leaf protein and, F-box family protein (Table 2). qPCR results showed that these genes were negatively correlated with the expression pattern of their complementary miRNA, with the exception of the putative target of miR171a-5p (methylketone synthase Ib). The unexpected behavior of the analyzed methylketone synthase gene might indicate that other regulatory mechanisms prevail over miR171 regulation, in the conditions of the present study (KPhi treatment), or that methylketone synthase is not a miRNA171 target in this system thus, miR171 might exert its action through other untested target genes.

The target genes analyzed in this work are mainly involved in plant defense mechanisms. For instance, it is known that bZIP transcription factors (miR166b targets) are involved in both abiotic and biotic stress responses [26]. Zhou *et al.* (2018) have described a potato bZIP transcription factor (StbZIP61) that regulates the biosynthesis of salicylic acid (SA) in the defense response against *P. infestans* [27]. Moreover, putative targets of miR482a-3p and miR482c PSH-RGH7 resistance and TIR-NBS-LRR resistance proteins, respectively, belong to the class of characterized disease resistance genes (R genes). Plant R genes encode nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins postulated to be involved in the detection of diverse specialized effectors from pathogens such as bacteria, viruses, fungi, nematodes, insects and oomycetes [28–31]. Despite the fact that in various plant species, computational analyses predicted a high number of genes regulated by miRNAs, a few of them were validated in potato. In this work, we validated in potato, one of the predicted targets genes for stu-miR530_L-2R+2, a Zinc Knuckle (CCHC-type) family protein. Zinc Knuckle are zinc finger proteins containing a characteristic motif with two short β-strands joined by a turn. The expression of a potato zinc finger protein gene, StZFP1, has been reported to increase upon biotic and abiotic stress, and after exogenous ABA application [32]. Although miR530 has been shown to target a zinc knuckle protein in rice [33], there are no previous reports available in potato. stu-MIR7985-p5 was differentially expressed after KPhi treatment and targeted a Spotted Leaf protein (SLp). Spotted Leaf proteins are U-box domain proteins that have been described to participate in the regulation of cell death and defense response mechanisms [34,35]. Zhang *et al.* (2013) have identified miR7985 in *Solanum tuberosum* by high-throughput sequencing [18], however, we report for the first time that this miRNA targets an SLp protein in potato.

We have validated the differential expression of PC-5p-1305_1307, a putative novel miRNA that was up-regulated after KPhi treatment. This potential miRNA targets, and negatively regulates an F-box protein. F-box proteins are key factors in phytohormone perception and stress signaling, photoperiodism, and metabolism regulation [36]. Our results are in accordance

with previous works that have reported, in Arabidopsis plants exposed to UV-B radiation, the suppressed expression of 4 F-box proteins, resulting in enhanced polyphenol levels, suggesting that they are involved in tolerance mechanisms against UV-B abiotic stress [37].

Interestingly, most of the predicted target genes for the potential novel miRNAs described in this work belong to genes involved in plant defense reactions (S4 Table) and might play an important role in the regulation of the molecular events mediated by KPhi. Further experiments such as the validation of the potential new miRNAs and, a degradome analysis must be performed to analyze the battery of target genes that are differentially expressed after KPhi treatment.

In order to gain information about the possible regulation of KPhi responsive miRNAs, the presence of well characterized cis-acting elements in the promoters of pre-miRNAs was analyzed *in-silico*. We focused on cis-elements involved in biotic and abiotic stress and phytohormone responsiveness. Noteworthy, cis-elements that respond to phytohormones, fungal elicitors and stress were found in the promoters of most pre-miRNAs. A few stress-related cis-elements were found in most of KPhi responsive miRNAs (ARE, MBS, TC rich repeat, I box, and G-box). Regarding phytohormone responsiveness, TCA, a cis-element responsive to phytohormones, was present in most differentially expressed miRNAs. Interestingly, stu-miR530_L-2R+2 has 8 light-responsive elements (G-box and I-box) and 5 ABA-regulated sites. stu-MIR7985-p5 has 4 abscisic acid inducible cis-elements (MBS), while miRNAs stu-miR166b, sly-miR159, stu-miR482c, stu-miR398b, and sly-miR167b have a Box-W1, a motif responsive to, a motif responsive to fungal elicitors and wounding.

The results presented in this work are consistent with the protective function of KPhi in potato described previously elsewhere. The diversity of differentially expressed miRNAs and the variety of cis-elements present in their promoters suggest that miRNAs are likely to participate in KPhi-dependent induction of a plethora of defense pathways [5–7,11,38–40]. It has also been reported that these responses are dependent on the action of phytohormones such as SA, jasmonic acid (JA), auxins, and ethylene [10,41,42]. The cross-talk between hormone signaling pathways in plants has been extensively documented [43,44]. In this scenario, phosphites might be a part of the intricate network of hormones/effectors activated as a defensive response upon stress.

In summary, miRNAs that respond to KPhi treatment have a wide range of functions in plants, as their predicted target genes. These results are consistent with the number and diversity of responses that KPhi triggers in potato. In this work, we provide evidence that the amplitude of responses associated with KPhi treatment can be, at least in part, explained by the diversity of miRNAs that are differentially expressed.

This is to our knowledge the first analysis of responsive miRNAs to a biocompatible stress resistance inducer as KPhi in potato. Additionally, we validated for the first time two predicted targets for potato miRNAs and a potential novel miRNA. Further characterization of these miRNAs and their target genes, might help to elucidate the molecular mechanisms underlying KPhi-induced resistance. This might in turn, aid in the design of genetically engineered potatoes to achieve a product with enhanced resistance to environmental stress.

Supporting information

S1 Table. miRNAs and target genes primers.
(DOC)

S2 Table. RNA seq library reads.
(XLS)

S3 Table. miRNAs differential expression.
(XLS)

S4 Table. Target prediction.
(XLS)

S5 Table. GO analysis.
(XLS)

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