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A Comprehensive Expression Profile of MicroRNAs and Other Classes of Non-Coding Small RNAs in Barley Under Phosphorous-Deficient and -Sufficient Conditions

Michael Hackenberg[1], Po-Jung Huang[2], Chun-Yuan Huang[3], Bu-Jun Shi[3,*], Perry Gustafson[4], and Peter Langridge[3,*]

Computational Genomics and Bioinformatics Group, Genetics Department, University of Granada, Granada 18071, Spain[1]; Bioinformatics Center, Chang Gung University, Taoyuan, Taiwan, China[2]; Australian Centre for Plant Functional Genomics, The University of Adelaide, Adelaide, South Australia 5064, Australia[3] and USDA-ARS, University of Missouri, 206 Curtis Hall, Columbia, MO 65211-7020, USA[4]

*To whom correspondence should be addressed. Tel. +(61) 8-83037168. Fax. +(61) 8-83037102.
Email: bujun.shi@acpfg.com.au (B.-J.S.); Tel. +(61) 8-83037014. Fax. +(61) 8-83037102.
Email: peter.langridge@acpfg.com.au (P.L.).

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Abstract

Phosphorus (P) is essential for plant growth. MicroRNAs (miRNAs) play a key role in phosphate homeostasis. However, little is known about P effect on miRNA expression in barley (*Hordeum vulgare* L.). In this study, we used Illumina's next-generation sequencing technology to sequence small RNAs (sRNAs) in barley grown under P-deficient and P-sufficient conditions. We identified 221 conserved miRNAs and 12 novel miRNAs, of which 55 were only present in P-deficient treatment while 32 only existed in P-sufficient treatment. Total 47 miRNAs were significantly differentially expressed between the two P treatments ($|\log_2| > 1$). We also identified many other classes of sRNAs, including sense and antisense sRNAs, repeat-associated sRNAs, transfer RNA (tRNA)-derived sRNAs and chloroplast-derived sRNAs, and some of which were also significantly differentially expressed between the two P treatments. Of all the sRNAs identified, antisense sRNAs were the most abundant sRNA class in both P treatments. Surprisingly, about one-fourth of sRNAs were derived from the chloroplast genome, and a chloroplast-encoded tRNA-derived sRNA was the most abundant sRNA of all the sRNAs sequenced. Our data provide valuable clues for understanding the properties of sRNAs and new insights into the potential roles of miRNAs and other classes of sRNAs in the control of phosphate homeostasis.

Key words: deep sequencing; barley; phosphorus; non-coding small RNAs; miRNAs; differential expression

1. Introduction

MicroRNAs (miRNAs) are a novel class of single-stranded, non-coding (nc) small RNAs (sRNAs). They are about 21–22 nucleotides (nt) in length, derived from hairpin precursors (pre-miRNAs) that in turn are formed from miRNA primary transcripts. MiRNAs play critical roles in the regulation of genes responsible for cell division, differentiation, development and signalling.[1] So far, 4053 miRNAs have been identified in plants, including 22 in barley (*Hordeum vulgare* L.) (miRBase version 18, November 2011).

Another important class of sRNAs is small interfering RNAs (siRNAs) that also regulate gene expression. Although siRNAs are similar in size and function to miRNAs, they are double-stranded (ds) and processed from perfectly long dsRNAs derived from viruses, transgenes or endogenous transcripts such as transposons.

Based on the genomic origin and the interaction of distinct components, endogenous siRNAs can be further classified into trans-acting siRNAs, natural antisense transcript-derived siRNAs (natsiRNAs), repeat-associated siRNAs (rasiRNAs), long siRNAs[2] and heterochromatin siRNAs.[3] All these subclasses of siRNAs are involved in biological processes.

Recently, several other classes of sRNAs were defined. They are tiny ncRNAs, 21U-RNAs, scan RNAs, promoter/termini-associated sRNAs, transcription initiation RNAs, transcription start site-associated RNAs, splice site RNAs and sRNAs derived from ribosomal RNAs (rRNAs), small nucleolar RNAs (snoRNAs) and transfer RNAs (tRNAs).[4,5] Most of these sRNAs are generated through the miRNA/siRNA pathway.[5] Distinctively, tRNA-derived sRNAs (tsRNAs) are essentially restricted to the cytoplasm and associated with argonaute proteins.

Barley is one of the most important cereal crops worldwide, ranking fourth in terms of production and is widely used for brewing and animal feed. Phosphorus (P) is an essential nutrient for plant growth and development. In cereal crops such as barley and wheat (*Triticum aestivum*), sufficient P in plants increases while deficient P in plants decreases the plant growth and grain yields.[6–8] Unfortunately, P availability in soil is low because soil P is often present in the precipitated or organic form that cannot be taken up by plants. On the other hand, plant requirements are high, especially for wheat and barley, whose production uses approximately 46% of the P fertilizers applied to cereals according to Food and Agriculture Organization (FAO) of the United Nations (FAO Fertilizer and Plant Nutrition Bulletin 17; http://www.fao.org). Thus, the improvement of P uptake from low concentrations of P in soil by cereal crops is crucial for sustainable agriculture and food security. Recently, miRNAs were found to participate in P uptake into plants.[9] While miRNAs regulate P starvation-responsive genes at the post-transcriptional level, they are regulated by different P concentrations *per se*. In lupin and soybean, 167 out of 771 and 57 out of 853 miRNAs examined, respectively, are induced by P limitation.[10,11] In *Arabidopsis,* miR399, miR778, miR2111 and miR827 are specifically induced by deficient P.[12] miR399 has been demonstrated to be a component of the shoot-to-root P deficiency signalling pathway.[13,14] In contrast to miR399, the roles of miR778, miR2111 or miR827 in the regulation of phosphate (Pi) homeostasis are not clear. In this study, we used Illumina's next-generation sequencing technology to sequence sRNAs in the shoots of barley grown under P deficient and P sufficient conditions. Our data reveal a comprehensive profile of miRNAs and other classes of sRNAs under different P conditions and provide a valuable resource for identifying miRNAs and/or sRNAs important for the control of Pi homeostasis.

2. Materials and methods

2.1. Barley cultivar

Pallas barley (*H. vulgare* L., cv Pallas) is an X-ray mutant widely used for plant breeding and evolution.[15] Pallas barley was grown in soil supplied with either 22.5 mg (deficient P) or 75 mg (sufficient P) Pi (KH_2PO_4) kg^{-1} dry soil. Basal nutrients and calcium carbonate were added into the soil, and growth conditions were the same as those described.[16] The plants were harvested 16 days after seed imbibition. It is to note that the P supply of 22.5 mg Pi in the soil only results in moderate P deficiency in barley plants. The reason we adopted such Pi concentration is based on realistic soil P-deficient condition and previous studies[8,17,18] showing that the supply of 22.5 mg Pi in the soil leads to 30% reduction in P concentration in barley shoots when compared with the critical P concentration required by barley plants.

2.2. sRNA isolation and sequencing

Total RNA was extracted from shoot material using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). sRNAs were isolated from total RNA using the Purelink miRNA Isolation Kit (Invitrogen, Carlsbad, CA, USA) and then electrophoresed on a 15% polyacrylamide (30:0.8) gel containing 7 molar (M) urea in 1× Tris-borate-ethylenediaminetetraacetic acid (EDTA) buffer (TBE: 0.04 M Tris base, 0.04 M acetate, 0.001 M EDTA, pH 8.0). Eighteen- to 30-nt sRNAs excised and eluted from the gel were ligated with both 5′ (5′-GUUCAGAGUUCUACAGUCCGACGAUC-3′) and 3′ (5′-pUCGUAUGCCGUCUUCUGCUUGUidT-3′, p-Pi, idT-inverted deoxythymidine) adaptors using T4 RNA ligase. Sequencing was performed using the 36-base Illumina platform.

2.3. Trimming of sRNA sequences and bioinformatics analysis

The sequence reads obtained from the 36-base Illumina platform were filtered and trimmed as follows: after removing poly A, T, C, G and/or N-containing reads, all reads with the same read sequences were grouped and termed 'unique reads'. Next, the 3′ adapter sequence was removed as described.[19] After adapter detection, the reads were re-grouped to calculate the read count of unique read sequences. All trimmed reads were compared with the complete barley chloroplast genome sequence without mismatches and then separated into non-chloroplast reads (nuclear-derived reads) and chloroplast reads.

The expression of known miRNAs and other transcribed sequences was analysed using the miRanalyzer standalone version.[19] Zero mismatch was set for all libraries except for Rfam that allows two mismatches. For all other parameters, the default values were used. After the reads were mapped to a given library, the mapped reads were removed and the remaining reads were used to map to another library and so on. To identify sRNAs derived from repeats such as (retro)transposons, the reads with a minimum length of 20 bp were aligned to the following libraries: (i) barley and rice libraries from the TIGR repeat database,[20] (ii) the Triticeae repeat sequence database (TREP)[21] and (iii) all RepBase repeats.[22] To identify sRNAs corresponding to rRNAs, tRNAs, small nuclear RNAs (snRNAs), snoRNAs, spliceosome RNAs (U1−U6) and other annotated ncRNAs, we compared the reads with sequences extracted from the Rfam database containing various ncRNA families and other structured RNA elements. To analyse antisense sRNAs (also called siRNAs), we first removed the reads mapped to known miRNAs and then aligned the remaining reads to the reverse strand of all barley genes available in the databases. Reads hitting the same genes were grouped into 'read clusters' based on their sequence similarity. The 'anti-sense' reads were then mapped to the forward strand of all other libraries (tRNA, genes, repeats, etc.). Because a gene could be hit by different reads (different length variants or from different genome elements), we clustered similar reads together (reads that are perfect inclusions of each other).

2.4. Prediction of novel miRNAs and their targets

Prediction of novel miRNAs was performed with miRDeep[23] that uses a probabilistic model of miRNA biogenesis to score compatibility of the position and frequency of the deep-sequencing reads with the hairpin structure of the miRNA precursor. All the trimmed reads were mapped (without mismatch) to barley expressed sequence tag (EST) sequences retrieved from the HarvEST:Barley (version 1.83) database (http://harvest.ucr.edu). The predicted precursor sequences were examined for their stem-loop structures with MFOLD.[24] Only putative miRNA precursors meeting a minimum free energy lower than −16 kcal/mol were retained for next analysis. Target genes of the putative novel miRNAs were predicted using psRNATarget,[25] a plant sRNA target analysis server (http://plantgrn.noble.org/psRNATarget).

2.5. Degradome library construction

A degradome library was constructed as described.[26] Briefly, poly(A) RNA, extracted from total RNA of Pallas shoots and roots using the Oligotex Kit (Qiagen), was ligated with a 5′ RNA adaptor containing a *Mme*I restriction site using T4 RNA ligase (Invitrogen), followed by reverse transcription, second-strand synthesis, *Mme*I digestion, ligation of a 3′ dsDNA adaptor, gel purification, and polymerase chain reaction (PCR) amplification. Amplified PCR products were sequenced with the Illumina HiSeq platform.

2.6. Northern blot hybridization

Fifty micrograms of total RNA extracted from barley were separated on a 15% polyacrylamide gel containing 7 M urea and transferred to Hybond-N membrane (Amersham Bioscience) using 20× saline-sodium citrate buffer (SSC: 3 M NaCl and 0.3 M sodium citrate, pH 7.0). The membrane was hybridized with ^{32}P-labelled DNA oligonucleotide probe reverse complementary to predicted miRNA sequence and made with γ-^{32}P -adenosine triphosphate (ATP) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA). A U6 snRNA-specific probe served as a loading control. Prehybridization and hybridization were both performed at 37°C in 125 mM Na_2HPO_4 (pH 7.2), 250 mM NaCl, 7% sodium dodecyl sulphate (SDS) and 50% formamide. Washing was also performed at 37°C using 2x SSC (0.3 M NaCl and 0.03 M sodium citrate), 0.2% SDS, twice followed by 1x SSC, 0.1% SDS once. The membrane was subjected to autoradiography at −80°C for 7 days.

2.7. Southern blot hybridization

Southern blot hybridization was carried out as described.[27] Briefly, barley genomic DNA was digested with appropriate restriction enzymes and separated via an electrophoresis gel. The DNA was then transferred to a membrane and hybridized with radiolabelled probes. After hybridization, the membrane was washed once at 65°C for 30−45 minutes (min) in 2x SSC containing 0.1% SDS and twice at 65°C for 30−45 min in 1x SSC containing 0.1% SDS. The membrane was subjected to autoradiography at −80°C as required until some background signal became visible.

2.8. Reverse transcription PCR (RT-PCR)

Reverse transcription PCR (RT-PCR) was performed as described.[28] First-strand cDNA was synthesized using total RNA as template with a primer reverse complementary to a miRNA precursor sequence. The synthesized cDNAs of pre-miRNAs were amplified using specific primer pairs with the conditions: 7 min at 95°C, followed by 45 cycles of 10 seconds (s) at 95°C, 10 s at 55°C and 30 s at 72°C and final extension step at 72°C for 10 min. The amplified products were electrophoresed in a 2% agarose gel in 1× Tris-acetate-EDTA buffer (TAE: 40 mM Tris base, 40 mM acetate and 1 mM EDTA, pH 8.3).

3. Results

3.1. *Phenotype of Pallas barley under P-deficient condition*

Pallas barley was grown in the soil supplied with 22.5 mg Pi kg^{-1} soil (moderately deficient P, hereafter designated deficient P) and 75 mg Pi kg^{-1} soil (sufficient P). Under deficient P, Pallas grew slowly when compared with plants grown with sufficient P, but showed no other symptoms of P deficiency (Fig. 1).

3.2. *Size distribution of sRNAs from P-deficient and P-sufficient barley shoots*

Totals of 7 006 282 sRNA sequencing reads of 36 bases from P-deficient barley shoots and 6 184 850 sRNA sequencing reads of 36 bases from P-sufficient barley shoots were obtained [the sequences have been deposited in the publicly accessible database, National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/sra) with accession number SRA025074.1]. Figure 2A shows the size distribution of sRNAs in P-deficient and P-sufficient shoots after the removal of poly A, T, C, G, N and adaptor sequences. Twenty and 21 nt were the two major size classes in each P treatment. Twenty four-nt sRNAs were abundant in P-deficient shoots, but not in P-sufficient shoots. Nineteen- and 22-nt sRNAs were slightly less abundant than the 20-, 21- and 24-nt sRNAs for both P treatments. Other sizes of sRNAs were found in low abundance in both P treatments. Overall, 19-, 20-, 21- and 24-nt sRNAs were more abundant in P-deficient shoots than in P-sufficient shoots (Fig. 2A). These data are not very consistent with the size distribution of sRNAs from another barley cultivar Clipper (*H. vulgare* L. cv Clipper), where 24 nt was the only major size class.[29] Clipper and Pallas are two distinct barley genotypes. Taken together, these suggest that the expression of 24-nt sRNAs is subject to conditions.

After the removal of duplicated reads, 24-nt sRNAs were the most dominant size class, followed by the 23-nt sRNAs for both P treatments (Fig. 2B). This indicates that 20- and 21-nt sRNAs largely consisted of redundant sRNAs, whereas 24-nt sRNAs primarily comprised unique or low abundant sRNAs. Twenty four-nt sRNAs, which are implicated to derive from intergenic and repetitive genomic regions and mainly guide transcriptional gene silencing,[30,31] accounted for 48.5% of the total unique sRNAs in P-deficient shoots and 29.4% in P-sufficient shoots. The number of 24-nt sRNAs in P-deficient shoots was over twice that in P-sufficient shoots. In contrast, the numbers of other sizes of sRNAs were similar for each P treatment (Fig. 2B). When a read count threshold $\geq$4 or 10 was applied, all sRNA sizes were significantly decreased in number in both P-deficient and P-sufficient shoots, except for 24-nt sRNAs in P-deficient shoots, indicating that most sRNAs are low copy.

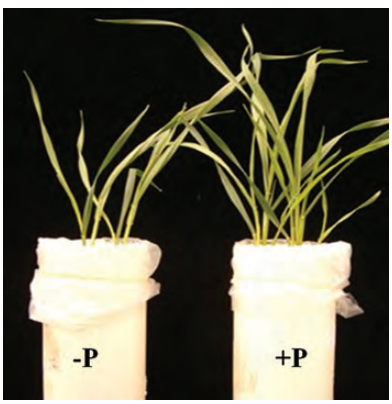

Figure 1. Phenotypes of barley (*H. vulgare* L. cv Pallas) grown for 16 days in soil amended with either 22.5 mg (deficient P) or 75 mg (sufficient P) KH_2PO_4 kg^{-1} dry soil.

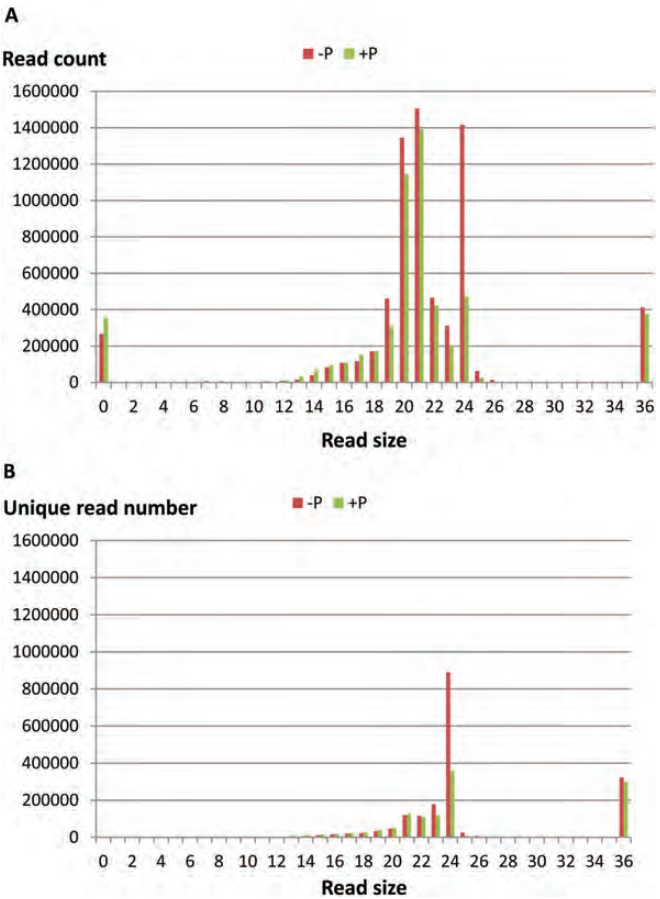

Figure 2. Size distribution of sRNA sequencing reads from P-deficient and P-sufficient barley shoots. (A) Size distribution of total reads. (B) Size distribution of unique reads.

3.3. *sRNAs are generated from both the nuclear and chloroplast genomes*

Chloroplasts have their own genome, transcriptional and translational machineries. Many chloroplast-derived sRNAs (csRNAs) have recently been identified.[32–34] We took advantage of the available barley chloroplast genome sequence to distinguish between sRNAs derived from the chloroplast and nuclear genomes in barley and found that about one-fourth of sRNAs were derived from the chloroplast genome in both P-deficient and P-sufficient shoots (Table 1). In contrast to sRNAs derived from the nuclear genome, csRNAs were highly redundant in both P treatments. Further analysis showed that there were fewer differentially expressed sRNAs derived from the chloroplast genome in P-deficient shoots than in P-sufficient shoots, whereas the trend was reversed for differentially expressed sRNAs derived from the nuclear genome (Table 1).

When a read count threshold ≥ 4 or 10 was applied, the numbers of P treatment-specific sRNAs derived from the nuclear or chloroplast genome were greatly reduced (Table 1), indicating that many of the treatment-specific sRNAs are low in abundance. The abundance of sRNAs derived from the nuclear or chloroplast genome, or common to both P treatments, is also reduced, but not significantly. In contrast, the abundance of sRNAs derived from the nuclear or chloroplast genome, or differentially expressed under P deficiency and sufficiency, is less changed, indicating that most of these sRNAs are relatively abundant.

Table 1. Statistics of the sequencing reads in P-deficient and P-sufficient shoots of Pallas barley

	Non-chloroplast reads			Chloroplast reads (0 mismatch)		
≥ 1	*Pallas* + P	*Pallas* − P	+P/−P	*Pallas*+P	*Pallas* − P	+P/−P
Unique reads	811 530	1 394 560	0.58	54 608	62 942	0.87
Total read count	3 153 867	4 137 948	0.76	1 153 778	1 738 653	0.66
Treatment-specific reads	650 912	1 233 942	0.53	31 604	39 938	0.79
Common reads		160 618			23 004	
	Down-regulated		Up-regulated	Down-regulated		Up-regulated
	25 981		27 132	6327		2050
	Percentage of dysregulated reads			Percentage of dysregulated reads		
	33.07			36.42		
≥ 4	*Pallas* + P	*Pallas* − P	+P/−P	*Pallas* + P	*Pallas* − P	+P/−P
Unique reads	36 309	68 306	0.53	7575	9857	0.77
Total read count	2 282 413	2 533 554	0.90	1 091 929	1 665 248	0.66
Treatment-specific reads	2314	5193	0.45	497	477	1.04
Common reads	42 104			7446		
	Down-regulated		Up-regulated	Down-regulated		Up-regulated
	9243		12 259	2698		1194
	Percentage of dysregulated reads			Percentage of dysregulated reads		
	51.07			52.27		
≥ 10	*Pallas* + P	*Pallas* − P	+P/−P	*Pallas* + P	*Pallas* − P	+P/−P
Unique reads	13 577	19 148	0.71	2503	3219	0.78
Total read count	2 157 072	2 270 506	0.95	1 063 737	1 628 357	0.65
Treatment-specific reads	96	330	0.29	11	62	0.18
Common reads	17 140			2938		
	Down-regulated		Up-regulated	Down-regulated		Up-regulated
	4293		3753	1032		487
	Percentage of dysregulated reads			Percentage of dysregulated reads		
	46.94			51.70		

≥ 1, ≥ 4 or ≥ 10 is the read count threshold. For the detection of down-regulated [$\log_2(-P/+P) \leq -1$] and up-regulated reads [$\log_2(-P/+P) \geq 1$], only common reads (present in both P conditions) are used.

3.4. *Expression profile of miRNAs in P-deficient and P-sufficient shoots*

To identify barley miRNAs, all non-chloroplast reads from P-deficient and P-sufficient shoots were aligned to known barley miRNAs from miRBase. Nineteen barley miRNAs were mapped in P-sufficient shoots and 18 in P-deficient shoots (Table 2, Supplementary Table S1). The extra miRNA in P-sufficient shoots is miR444a that is a low-copy miRNA. It is unclear if this miRNA is a *bona fide* miRNA or resulted from a sequencing error. Three barley miRNAs, hvu-miR399, hvu-miR1126 and hvu-miR5051, were significantly up-regulated in P-deficient shoots ($\log_2 > 1$) (Table 2, Supplementary Table S1). The expression pattern of miR399 is consistent with previous reports,[13] suggesting that these miRNAs may indeed be up-regulated by deficient P. Hvu-miR168 was the most abundant barley miRNA, followed by hvu-miR156 in both P treatments (Table 2, Supplementary Table S1). The two miRNAs together accounted for 95.3% of the annotated barley miRNAs in P-sufficient shoots and 95.1% in P-deficient shoots. Such highly expressed miRNAs are likely to have important roles in barley. In *Arabidopsis*, miR168 has been shown to regulate argonaute-1 (AGO1) that is involved in both miRNA biogenesis and mRNA degradation,[3] whereas miR156 regulates squamosa promoter binding protein-like (SPL) transcription factors that define an endogenous flowering pathway.[35] However, neither of these two miRNAs was significantly differentially expressed between P-deficient and P-sufficient shoots, suggesting that the expression of these two miRNAs may be independent of P nutritional status. To identify conserved miRNAs, the remaining unmapped reads were aligned to miRNAs from other species in miRBase. One hundred and forty seven miRNAs in P-sufficient shoots and 171 in P-deficient shoots were mapped (Supplementary Table S2). Of these conserved miRNAs, 55 were only present in P-deficient shoots while 32 only existed in P-sufficient shoots. MiR156 was the most abundant among all the conserved miRNAs identified in both P treatments.

Seven conserved miRNAs were up-regulated, while 19 were down-regulated ($|\log_2| > 1$) in P-deficient shoots when a threshold was ≥ 10 (Table 3). MiR399 was only detected in P-deficient shoots, but not in P-sufficient shoots (Table 3). MiR827 was also significantly up-regulated, being 8 times more abundant in P-deficient shoots than in P-sufficient shoots, which is consistent with previous studies.[12,36] In contrast to miR399, miR827 was much more abundant in both P treatments (Table 3). In addition, miR827 has an additional role in drought tolerance (M. Aukerman and W. Park, unpublished data). These suggest that the two P-responsive miRNAs might be regulated by different

Table 2. Barley miRNAs identified in P-deficient and P-sufficient shoots of Pallas barley

Name	Read count +P	Read count −P	−P norm	Log_2
hvu-miR168-5p	511 386	533 602	406 701.5194	−0.330442284
hvu-miR156	307 838	223 346	170 230.167	−0.854684607
hvu-miR166/b/c	34 661	31 173	23 759.48078	−0.544809969
hvu-miR5048	2900	3146	2397.822685	−0.274327923
hvu-miR159a/b	2248	3011	2294.928196	0.029806979
hvu-miR168-3p	295	327	249.2333178	−0.243218012
hvu-miR1120	172	198	150.9119172	−0.188701828
hvu-miR5049	155	134	102.1323076	−0.601828908
hvu-miR171	152	182	138.7170148	−0.131926566
hvu-miR444b	73	51	38.8712514	−0.90919291
hvu-miR5052	51	47	35.8225258	−0.509630184
hvu-miR397	48	46	35.0603444	−0.453194238
hvu-miR1126	24	64	48.7796096	1.023243806
hvu-miR5050	14	13	9.908358201	−0.498708897
hvu-miR1436	10	12	9.146176801	−0.128759287
hvu-miR399	6	377	287.3423878	5.581664519
hvu-miR5053	3	2	1.5243628	−0.976756194
hvu-miR5051	2	8	6.0974512	1.608206307
hvu-miR444a	1	0	0	#NUM!

'#NUM' means that the $\log_2$ cannot be calculated with a read count of 0 in either sample. '−P norm' is a linear normalization to get the total read counts equal in both samples. The same meaning below.

Table 3. Differentially expressed miRNAs between P-deficient and P-sufficient shoots of Pallas barley

Name	Read count +P	Read count −P	−P norm	Log_2
zma-miR156k/sbi-miR156e	1334	508	387.18815	−1.78465
osa-miR827a/b/zma-miR827/bdi-miR827/ssp-miR827	1152	9344	7121.823	2.628106
ptc-miR156k/vvi-miR156a	710	455	346.79254	−1.03375
bdi-miR408	247	85	64.785419	−1.93077
rgl-miR5139	155	100	76.21814	−1.02406
vvi-miR156e	100	64	48.77961	−1.03565
ath-miR165a/b	85	22	16.767991	−2.34175
mtr-miR156j	83	48	36.584707	−1.18187
vvi-miR167c	64	34	25.914168	−1.30433
bdi-miR166e	58	34	25.914168	−1.16231
zma-miR396a*/b*	51	21	16.005809	−1.6719
ath-miR399b/c/d/sbi-miR399d/i/zma-miR399e/i/j/mtr-miR399l/p/vvi-miR399b/c/pvu-miR399a/rco-miR399a/aly-miR399b/c/csi-miR399d/tcc-miR399i/vun-miR399a	46	147	112.04067	1.284317
osa-miR5072	43	21	16.005809	−1.42574
csi-miR172b	28	17	12.957084	−1.11169
ghr-miR156c	21	13	9.9083582	−1.08367
zma-miR169i*/k*/j*	20	13	9.9083582	−1.01328
zma-miR156l*	19	8	6.0974512	−1.63972
bta-miR-2487	16	4	3.0487256	−2.39179
ptc-miR164f/mtr-miR164d/vvi-miR164b/rco-miR164d/tcc-miR164c	15	9	6.8596326	−1.12876
osa-miR399a/b/c/sbi-miR399a/c/h/j/zma-miR399a/c/h/ptc-miR399f/g/vvi-miR399a/h/csi-miR399e/c/tcc-miR399g/bdi-miR399b/vun-miR399b	11	148	112.80285	3.358228
osa-miR166e	11	4	3.0487256	−1.85123
zma-miR166b*/d*	10	5	3.810907	−1.39179
dme-miR-1-3p/dps-miR-1/ame-miR-1/aga-miR-1/bmo-miR-1a/tca-miR-1-3p/dan-miR-1/der-miR-1/dgr-miR-1/dmo-miR-1/dpe-miR-1/dse-miR-1/dsi-miR-1/dvi-miR-1/dwi-miR-1/dya-miR-1/dpu-miR-1/isc-miR-1/aae-miR-1/cqu-miR-1/api-miR-1/nvi-miR-1/ngi-miR-1	7	266	202.74025	4.856134
ame-miR-3772	4	28	21.341079	2.415561
tae-miR1126	2	12	9.1461768	2.193169
zma-miR399h*	0	145	110.5163	#NUM

Threshold ≥ 10 is applied. '−' indicates that miRNAs are down-regulated, whereas the others are up-regulated.

mechanisms. Surprisingly, one miRNA, dme-miR-1−3p, from *Drosophila* was detected and significantly up-regulated in P-deficient shoots (Table 3). MiRNAs conserved between plants and animals have been reported previously.[37] If this result is true, then dme-miR-1−3p could be used as a phylogenetic marker to help unravel the evolutionary history and relationships between plants and insects.

Previous studies showed that miR778 and miR2111 are up-regulated under P limitation in *Arabidopsis*.[12] However, these two miRNAs were not present in barley shoots, or in rice,[12] suggesting that they may be dicot specific. MiR398 was not present in barley shoots either, but it is present and down-regulated under P limitation in *Arabidopsis*.[12] In addition, unlike in *Arabidopsis*,[12] miR169 and miR395 were not down-regulated under P limitation in barley shoots (Table 3). These data indicate that miRNAs have different responses to P in different species.

To confirm the expression of conserved miRNAs, Northern hybridization was performed using probes reversely complementary to hvu-miR156, hvu-miR399, osa-miR528, bdi-miR827 and bdi-miR408. These miRNAs were selected because they were relatively abundant. Hvu-miR399 and bdi-miR408 were hardly detectable, but the three other miRNAs were detected in both P-deficient and P-sufficient

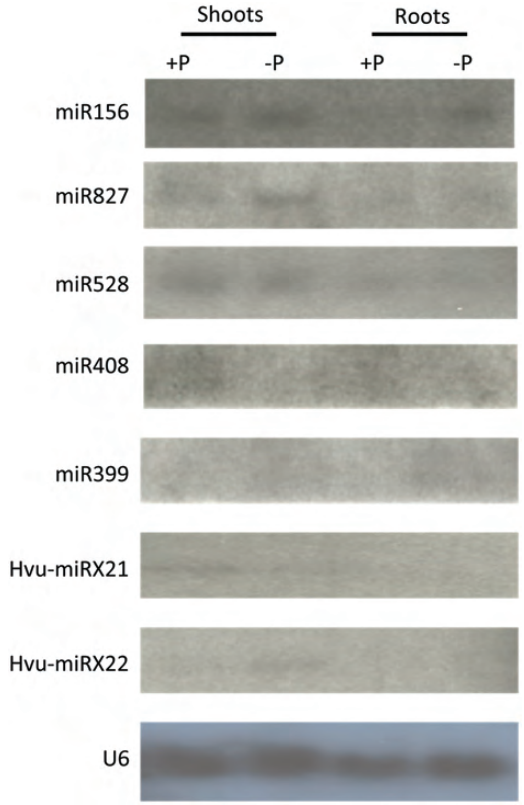

Figure 3. Northern blot hybridization of miRNA expression in P-deficient and P-sufficient shoots of Pallas barley. Total RNA extracted from leaf and root tissues from P-deficient (−P) and P-sufficient (+P) barley was hybridized with probes reversely complementary to indicated known and novel miRNAs. Hybridization with U6 probe was used as a loading control.

shoots (Fig. 3). Bdi-miR827 was expressed at a higher level in P-deficient shoots than in P-sufficient shoots, whereas osa-miR528 expression was slightly reduced under P deficiency (Fig. 3). Both miRNAs were expressed at higher levels in leaves than in roots (Fig. 3). The expression level of hvu-miR156 did not differ between P-deficient and P-sufficient leaves, but was higher in P-deficient roots than in P-sufficient roots (Fig. 3).The expression levels of these miRNAs detected by the Northern hybridization were in agreement with the read abundances of the same miRNAs identified from the deep sequencing.

3.5. *Predicted novel miRNAs in P-deficient and P-sufficient shoots*

To identify novel miRNAs, we only selected miRNA precursor sequences with secondary structures having higher negative minimal free energies and minimal free energy index (MFEI) than other different type of RNAs and MFEI of greater than 0.85.[38] Then, we aligned all reads to these selected miRNA precursor sequences. If the reads displayed one or two peaks without appearance in the intervening region, these reads would be taken on board as novel miRNA candidates for further analyses. This is based on the alignment patterns of known miRNAs from miRBase (data not shown). These two typical patterns can be explained by the stability of duplexes or the stability of miRNAs that are incorporated into the RNA-induced silencing complex (RISC). Accordingly, we identified 12 novel miRNA candidates present in both P-deficient and P-sufficient shoots (Table 4). One miRNA (hvu-miRX27) was found to exist in two mature sequences, but differing only by 1 nt extension at the ends (Table 4). It is unclear which mature sequence is a true miRNA sequence.

Most of the predicted novel miRNAs were 20–21 nt in length and had a preference for A at the 5′ end (Table 4), which is different from conserved miRNAs that have a preference for U at the 5′ end.[28,39,40] Most of the predicted novel miRNAs were expressed at higher levels in P-deficient shoots than in P-sufficient shoots (Table 4). Three miRNAs (hvu-miRX25, hvu-miRX29 and hvu-miRX32) were significantly up-regulated in P-deficient shoots (Table 4).

3.6. *Validation of predicted novel miRNAs*

Northern blot hybridization was used to validate the predicted novel miRNAs. Two (hvu-miRX21 and hvu-miRX22) out of 3 selected novel miRNA candidates (hvu-miRX21, hvu-miRX22 and hvu-miRX27) generated a clear hybridization signal of expected size in leaves, but not in roots, for both P treatments (Fig. 3). RT-PCR was used to further confirm the miRNA candidates. All the candidates generated specific products of expected size in shoot tissue (Fig. 4). Hvu-miRX27 appeared to be more highly expressed in P-sufficient shoots than in P-deficient shoots, whereas hvu-miRX21 showed similar yields between P-deficient and P−sufficient shoots.

3.7. *Predicted miRNA targets and validation*

MiRNA targets were predicted using psRNAtarget. All the novel miRNA candidates had their targets found in the HVGI database (Release 11). Some targets had known function, whereas others were not annotated (Table 4). In addition, some targets were targeted by abundant miRNAs, whereas others were targeted only by scarce miRNAs (Table 4). To validate the predicted targets, which could in turn confirm the predicted novel miRNAs, we constructed a degradome library based on the miRNA-guided cleavage of the target between the 10th and 11th nt of complementarity relative to the guiding miRNA. This library has advantages over the 5′-RACE approach that requires *a priori* miRNA target prediction and only tests one target at a time. This library has been deposited in NCBI with accession number

Table 4. Predicted novel miRNAs in P-deficient and P-sufficient shoots of Pallas barley by miRDeep

miRNA	Count −P	Count +P	Precursor miRNA	Target Accession	Action	Function
hvu-miRX21	3512	3279	U36_28540: 318.. 428: -, MFE= -31.30, aaacga--| a a ga uauc ccgcccu cacaggcu \ AUAG GGCGGGA gugucugg u CUUUUACA^ G G gu	TC207745	Cleavage	E3 ubiquitin-protein ligase
hvu-miRX22	3494	2015	U36_21163: 86..195: +, MFE= -35.40, AACU--| A au cuc UGU GAGGUGAUUUGGUg caccgaau \ aca cuucacuaaaccac guggcuua u uuucgu^ c ac ugu	TC236060	Cleavage	unknown
hvu-miRX23	85	69	U36_40128:251..339:+, MFE= -17.30, UGACC- G C C---- ua-| ua CUGAG CA UCAUAC Gc cc g gacuc gu aguaug ug gg u gcgauu g a ccauu uaa^ uu	TC224987	Translation inhibition Cleavage (two sites)	Homeodomain protein
hvu-miRX24	212	172	U36_48611:500..573:+, MFE= -27.70, A-- A -| GA g ucuu AUU AUUU GGAUCG GGGAguag ucau a uag uaaa ucuagc cccucauc agua u ucg a u^ ua a ucgu	TC195197	Cleavage	Chloroplast Mg-chelatase subunit XANTHA-G precursor
hvu-miRX25	28	12	U36_38416:101..176:-, MFE= -29.30, ---- uu -----| cuua gu ucugcccccua ccuc ugcg uuugug g GGAUGGGGGAU GGAG augu aaacac u CCAG C- UCUAu^ uua- gc	TC236049	Cleavage	Transcription regulator protein
hvu-miRX26	497	543	U36_57207:221..303:+, MFE= -54.60, U CUA g --| a GAUGUCCCAUCGAAGU Augaugccccac aga ag g cuacaggguggcuuca uacuacggggug ucu uc c a aac g ca^ u	DN159928	Translation inhibition Cleavage	Adhesion protein PapA
hvu-miRX27	230	131	U36_10756:49..127:+, MFE= -25.80, -- a c c -| u aac uccguuccaaaa aa uguuu aa cuu guauaa \ AGGCAGGGUUUU UU ACAaa uu gaa cauguu a GG A C a c^ u aaa	BQ756036	Cleavage	Terpene cyclase
hvu-miRX27	257	182	U36_10756:48..128:+, MFE= -28.90, -- a c c -| u aac cuccguuccaaaa aa uguuu aa cuu guauaa \ GAGGCAGGGUUUU UU ACaaa uu gaa cauguu a GG A C a c^ u aaa	TC196145	Cleavage (two sites)	CTR1-like kinase kinase kinase-like
hvu-miRX28	151	133	U36_68058:15..76:+, MFE= -16.19, CAG| CU GG aucuaag AAGAG GGGG UCGUCaa a uucuu uucc agcaguu c aag^ u- ag cuuaacu	TC229652	Cleavage	Unknown
hvu-miRX29	1463	765	U36_44241:58..107:-, MFE= -27.80, c C U UAUAG Aau auuu auuacggacuauAUA GGA GUA ACAU u uaag ugaugccuggguguau ucu cgu ugug u u - u uauaa aaa	TC195723	Cleavage	Pathogenesis-related protein 4 precursor
hvu-miRX30	585	392	U36_39963:452..512:+, MFE= -21.70, --| AA AGG ua AUAUUUAGG CGG GAGUauuuau a uauaaaucc gcc uucguaggua u ua^ ca a-- ug	TC214946	Cleavage	Phospholipid hydroperoxide glutathione peroxidase-like protein

Continued

Table 4. Continued

miRNA	Count −P	Count +P	Precursor miRNA	Target Accession	Action	Function
hvu-miRX31	360	269	U36_14667:271..361:-, MFE= -49.12, g aguacaaauu g ccuccguccaaaauaacugucuuaa cuu uauacua a GGAGGCAGGGUUUUAUUGauagaguu gaa auaugau u GAG^ - ---------- c	TC196806	Cleavage (four sites)	AMP-binding enzyme family protein expressed
hvu-miRX32	193	92	U36_59470:2..83:+, MFE= -44.70, A G -) acg - cu AC AGCCGGGACUAAAGGU Cu cc uggg cgc c ug uuggcccugauuucca ga gg accu gcg g gcacau c g c^ ga- u au	EX593121	Translation inhibition Cleavage	Unknown

'Count−P' and 'count +P' indicate read counts from P-deficient and P-sufficient barley shoots, respectively. Targets were predicted using psRNAtarget (http://plantgrn.noble.org/psRNATarget/). Cleavage sites were determined using degradome library.

SRS376178. Three predicted novel miRNAs, hvu-miR23, hvu-miR27 and hvu-miR31, out of 12 from P-deficient and P-sufficient shoots were perfectly reverse complementary to the first 10 nt of 3 sequence fragments in the library (Table 4), providing potential evidence of miRNA-mediated cleavage. Intriguingly, all these three target genes were cleaved more than twice at different positions (Table 4). This suggests that these miRNAs may have diverted cleavage sites. It is of notice that one target (DN159928) is regulated by the miRNA at both translational and transcriptional levels (Table 4).

3.8. *Profiles of other classes of nuclear-derived ncRNAs in P-deficient and P-sufficient shoots*

To explore the profiles of other classes of nuclear-derived ncRNAs in Pallas P-deficient and P-sufficient shoots, all the non-miRNA reads were aligned to the Rfam database. Seven hundred and ninety six and 715 ncRNAs were mapped in P-deficient and P-sufficient shoots, respectively, of which 114 ncRNAs were specific to P-deficient shoots, whereas 32 ncRNAs were specific to P-sufficient shoots (Supplementary Table S3). Of 683 shared ncRNAs, 176 were up-regulated whereas 51 were down-regulated in P-deficient shoots (Supplementary Table S3). The most abundant ncRNAs were small subunit rRNA 5-derived sRNAs, followed by tsRNAs in both P treatments. The two classes of sRNAs accounted for 80% of the mapped ncRNAs in P-deficient shoots and 76.3% in P-sufficient shoots (Supplementary Table S3).

tsRNAs were further dissected by aligning all the non-miRNA reads to tRNA databases. Fifty-six out of the total 61 tRNAs were found to generate tsRNAs in both P-deficient and P-sufficient shoots. These 56 tRNAs specify all essential 20 amino acids encoded by the standard genetic code (Supplementary Table S4). Six tsRNAs were significantly up-regulated ($\log_2 > 1$), whereas 4 were significantly down-regulated ($\log_2 < -1$), in P-deficient shoots (Supplementary Table S4). Notably, the read count of tsRNAs was 2-fold greater in P-deficient shoots than in P-sufficient shoots (Supplementary Table S4).

tsRNAs derived from tRNA-Gly(TCC) were the most abundant tsRNA species in both P treatments, accounting for 58.6% of the total tsRNAs in P-deficient shoots and 58.2% in P-sufficient shoots (Supplementary Table S4). tRNA-Ala(AGC)-derived sRNAs were the second most abundant species in P-sufficient shoots, but the third in P-deficient shoots. The second most abundant tsRNA in P-deficient shoots was tRNA-Arg(CCT)-derived sRNAs (Supplementary Table S4). The relative proportions of the other tsRNAs did not appear to be consistent between the two P treatments (Supplementary Table S4).

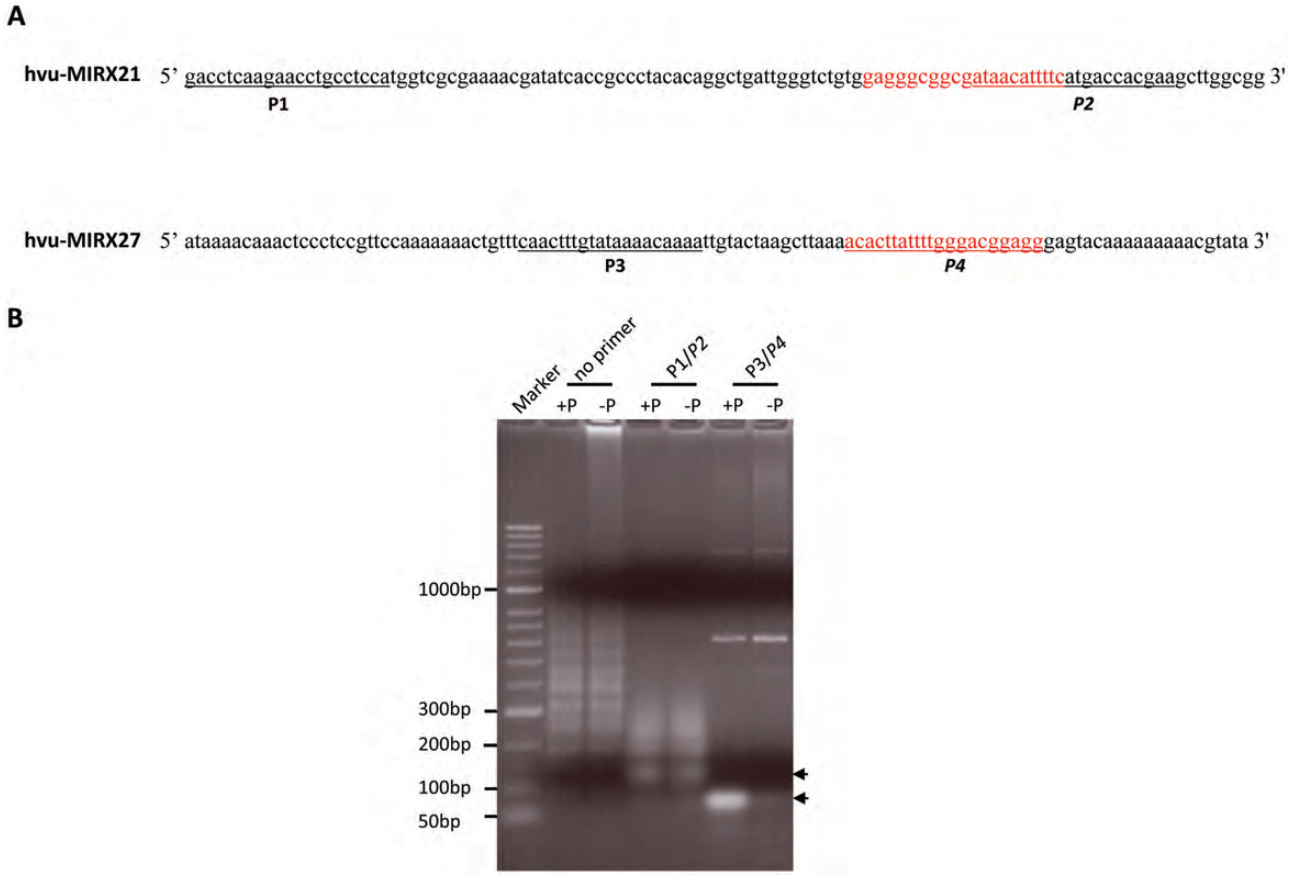

Figure 4. RT-PCR expression analysis of predicted novel miRNAs. (A) Sequences were used to amplify predicted novel miRNA precursor sequences with primers (underlined). Mature miRNA sequences are highlighted in red. P1 and P3 are forward primers while P2 and P4 are reverse primers indicated in Italics. (B) Amplification of the cDNAs synthesized from total RNA extracted from P-deficient (−P) and P-sufficient (+P) shoots was performed with primers as shown in (A). The samples, primers and sizes of RT-PCR products are all indicated.

Next, we analysed rasiRNAs by aligning all the non-miRNA reads to the TREP database that contains 1716 transposable elements (TEs). TEs are a major source of siRNAs[41,42] that in turn suppress the mutagenic activity of TEs.[43] The sRNAs derived from 685 TEs in P-deficient shoots and the sRNAs derived from 582 TEs in P-sufficient shoots were identified (Supplementary Table S5). Of them, 581 TE-derived sRNAs were common between the two P treatments. Almost every TE-derived sRNA was less abundant in P-sufficient shoots than in P-deficient shoots, with half of these significantly down-regulated ($\log_2 > -1$) (Supplementary Table S5). There are 59.8% of the TE-derived sRNAs being mapped to retrotransposons (Class I TEs), 32.6% to DNA transposons (Class II TEs) and 7.6% to unclassified TEs in P-sufficient shoots (Table 5). Coincidentally, 53% of the TE-derived sRNAs were mapped to retrotransposons, 39.6% to DNA transposons and 7.3% to unclassified TEs in P-deficient shoots (Supplementary Table S5). The percentage of each class of TE-derived sRNAs was similar between the two P treatments. Similar results were also obtained when the reads were aligned to other repetitive databases such as Repbase, the TIGR rice repeat database and the TIGR barley repeat database (Supplementary Table S5).

To analyse natsiRNAs, another source of siRNAs that are capable of blocking the translation of mRNAs or degrading mRNAs through the siRNA pathway,[44] all non-miRNA reads were firstly aligned to the reverse strand of barley genes available in the databases, and the mapped reads were then aligned to the forward strand of all other libraries, including tRNA, genes, repeats and so on. In total, 290 234 and 303 953 natsiRNAs were identified in P-deficient and P-sufficient shoots, respectively, of which many were treatment specific (Supplementary Table S6).

Some natsiRNAs were found to target multiple genes, whereas others target only a single gene (Supplementary Table S6). The latter accounted for 80.6% of the total mapped genes in P-deficient shoots and 78.2% in P-sufficient shoots. A great number of natsiRNAs were derived from TEs (Supplementary Table S6), which is consistent with

Table 5. Summary of mapped TE-derived sRNAs in P-deficient and P-sufficient shoots of Pallas barley

Name	Read count +P	Read count −P	−P norm	Log_2
Retrotransposon	4543	8934	6809.329	0.583867
DNA transposon	2482	6683	5093.658	1.037199
Unknown	577	1234	940.5318	0.704905
LTR	4366	8473	6457.963	0.564767
TIR	2469	6660	5076.128	1.039802
Unknown	582	1238	943.5806	0.697127
LINE	167	436	332.3111	0.992686
SINE	10	25	19.05454	0.930134
Helitron	8	19	14.48145	0.856134
Copia	2421	4580	3490.791	0.527951
Gypsy	1843	3599	2743.091	0.573746
CACTA	1387	3316	2527.394	0.865683
Unknown	861	1993	1519.028	0.819063
Mariner	559	1432	1091.444	0.965318
Harbinger	414	1616	1231.685	1.572931
Mutator	103	282	214.9352	1.061257
Helitron	8	19	14.48145	0.856134
HAT	6	14	10.67054	0.830599

previous studies.[45] Many natsiRNAs formed duplexes (Supplementary Table S6).

The discovery of siRNA duplexes prompted us to investigate sense siRNAs. Alignment of all the nuclear-derived reads to barley genes in the HVGI database identified 128 corresponding barley genes in P-sufficient shoots and 137 in P-deficient shoots (Supplementary Tables S5 under non-chloroplast sense sRNAs). Some genes had more associated reads than other genes. In addition, more sense siRNAs were present in P-deficient shoots than in P-sufficient shoots. The expression levels of sense siRNAs were also higher in P-deficient shoots than in P-sufficient shoots.

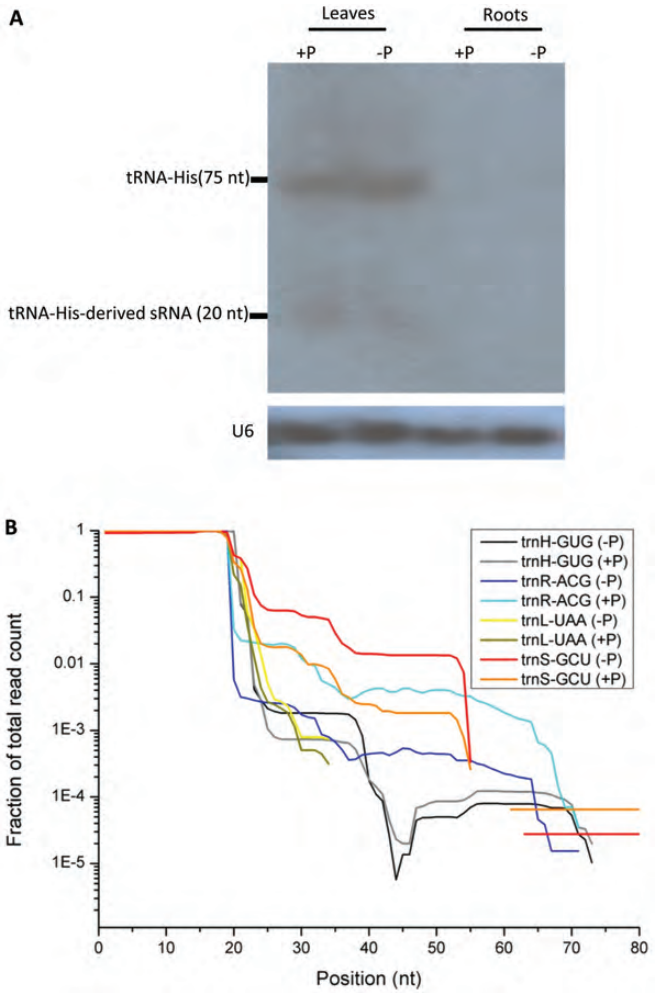

Figure 5. Northern blot hybridization of tsRNAs derived from tRNA-His(GTG) and read distribution on four tRNA genes in P-deficient (−P) and P-sufficient (+P) shoots of barley. (A) Total RNA extracted from leaf and root tissues from P-deficient and P-sufficient barley was hybridized with probes reversely complementary to the most abundant tsRNA derived from tRNA-His(GTG). Hybridization with U6 probe is used as a loading control. (B) All the reads obtained were aligned with each of the four most abundant tRNA genes. The names of tRNA genes, positions of reads on the tRNA genes and read fraction are indicated.

3.9. Profile of csRNAs in P-deficient and P-sufficient shoots

CsRNAs in the two P treatments were further analysed in detail. The result showed that the csRNAs were derived from all regions of the chloroplast genome, including CDS (Supplementary Table S7 under chloroplast sRNAs_CDS), tRNA genes (Supplementary Table S7 under chloroplast sRNAs_tRNA) and repetitive sequences (Supplementary Table S7 under chloroplast sRNAs_rep). The chloroplast-derived tsRNAs were the most abundant sRNA class, accounting for 22.7% of the total sRNAs in P-deficient shoots and 19% in P-sufficient shoots (Supplementary Table S7 under chloroplast sRNAs_tRNA). One tsRNA derived from the tRNA-His(GUG) gene was the most abundant of all the sRNAs (including miRNAs) sequenced. This tsRNA accounted for 84.5% of the total tsRNAs identified and 51.2% of the total csRNAs. This result explains why csRNAs are highly redundant when compared with sRNAs derived from the nuclear genome. Curiously, this most abundant tsRNA was only present in barley leaves and not in roots (Fig. 5A) and is perfectly reversely complementary to a gene involved in biological processes. The absence or low expression of this tsRNA in the roots may be due to reduced plastid copy numbers in roots compared to those in leaves.[46]

Alignment of all the csRNAs to the tRNA-HIS(GUG) gene revealed that the distribution of csRNAs over the gene was uneven. A peak was located at the 5′ end, where the sequence corresponded to that of the most abundant tsRNA (Fig. 5B), suggesting that the most abundant tsRNA is not randomly generated. Other tsRNAs gave a similar distribution pattern as the tRNA-HIS(GUG)-derived sRNAs (Fig. 5B).

tsRNAs derived from four tRNA genes, trnR-ACG, trnE-UUC, trnL-CAA and trnL-UAA, were significantly up-regulated ($\log_2 > 1$), whereas tsRNAs derived from the trnS-GCU gene were significantly down-regulated ($\log_2 < -1$), in P-deficient shoots (Supplementary Table S7 under chloroplast sRNAs_tRNA). A few other csRNAs derived from the other regions were also found to be significantly differentially expressed ($|\log_2| \geq 1$) between the two P treatments (Supplementary Table S7). These results imply that P nutrition is associated with the expression of some csRNAs.

Unexpectedly, 95 barley genes were mapped by csRNAs in each of the P-deficient and P-sufficient shoots (Supplementary Table S7 under chloroplast sRNAs_HVGI), although this number is lower than that mapped by the nuclear sRNAs (128 in P-sufficient shoots and 137 in P-deficient shoots). One barley gene (TC277237), encoding photosystem II reaction centre protein H, was mapped the most by csRNAs in both P treatments. sRNAs derived from seven barley genes were present in both the chloroplast genome and the nuclear genome but were differentially expressed between the two genomes (Table 6). For example, sRNAs derived from the gene TC250483 were more abundant in the nuclear genome than in the chloroplast genome, whereas sRNAs derived from the gene BY843503 were more abundant in the chloroplast genome than in the nuclear genome (Table 6). These data suggest that a genome or organelle-specific factor may be involved in the expression of each class of sRNAs.

4. Discussion

P is a key determinant of plant growth and development, but its availability is low in the soil. Therefore, finding an effective way to enable cereal crops to take up Pi from low P soils efficiently is desirable. MiRNAs have been shown to be important gene regulators in plant Pi homeostasis. In *Arabidopsis*, a few of miRNAs have been identified to be regulated by P,[47] suggesting a close relationship between miRNA and P. To investigate this relationship in barley, we comprehensively analysed sRNA sequence datasets generated by deep sequencing from Pallas barley grown under P-deficient and P-sufficient conditions. Our result showed that about 1 million sRNA sequences out of the total 7 million sequences were specific to either P-deficient or P-sufficient treatment. In addition, over 30% of common sequences were significantly differentially expressed between the two P treatments. These indicate that P has a great impact on the expression of sRNAs. How P affects the expression of sRNAs is unclear, but it likely involves its signalling pathway that results in some genes being activated, whereas others being degraded, under different P conditions, thus leading to different sRNA populations. We observed that more sRNAs were generated under P-deficient condition than under P-sufficient condition. A similar situation has been described previously.[48] In addition, we observed that the number of individual siRNAs or miRNAs is also more in P-deficient treatment than in P-sufficient treatment. Coincidentally, more mRNA-derived individual sRNAs were found in P-deficient treatment when compared with those in P-sufficient treatment. One model could be used to explain these phenomena: P deficiency causes the instability or degradation of RNA molecules firstly because P is a part of an RNA molecule, and the lack of P reduces its incorporation into the RNA molecules, and then the generated sRNAs are used as siRNAs to guide the cellular RNA degradation machinery.[49] This has been the case in

Table 6. Shared gene-derived sRNAs between chloroplast and nucleus

Mapped barley genes from nuclear					Mapped barley genes from chloroplast				
Name	Read count +P	Read count −P	−P norm	Log_2	Name	Read count +P	Read count −P	−P norm	Log_2
TC250483	235	226	172.253	−0.44813	TC250483	20	35	23.22616	0.215751
BY848620	31	35	26.67635	−0.21671	BY848620	111	73	48.44313	−1.1962
BM097437	20	21	16.00581	−0.3214	BM097437	112	84	55.74278	−1.00664
BY841253	17	10	7.621814	−1.15733	BY841253	526	312	207.0446	−1.34512
BY843503	14	34	25.91417	0.888314	BY843503	1329	2281	1513.682	0.187721
TC272545	10	11	8.383995	−0.25429	TC272545	25	265	175.8552	2.814388
TC279709	7	10	7.621814	0.122779	TC279709	18	21	13.9357	−0.36921

Drosophila[50] and *Arabidopsis*.[51] However, it must be pointed out that such degradation mode is only for sRNAs derived from the 3′, but not 5′, terminal regions.[45] The 5′ terminal degradation mode from the miRNA/siRNA-induced cleavage has not yet been elucidated so far.

We have identified a total of 233 miRNAs in Pallas barley, of which some were significantly differentially expressed between the two P treatments ($|\log_2| \geq 1$), whereas others were P treatment specific. A total of 214 miRNAs have been detected using cross-species comparison or prediction. In a recent study, 126 conserved miRNAs and 148 barley-specific miRNAs were identified in barley cultivar Clipper.[29] To add evidence to the identified miRNAs, we compared our set of miRNAs with the ones detected in Clipper. We found that of the total 233 miRNAs identified in Pallas, 94 (40.3%) have also been detected in Clipper (allowing at most 1 mismatch). There is a clear tendency between coexistence (expressed in both Pallas and Clipper) and expression level. The most expressed miRNAs in Pallas are also highly expressed in Clipper, whereas lower expressed miRNAs have a lower probability to co-exist.

Many miRNAs are known to show a strongly tissue-dependent expression profile. Because the Pallas and Clipper data were generated from different tissues (shoots only in Pallas and a mixed tissues of leaves, stems, roots and spikes in Clipper), the absence of co-existence does not necessarily imply a false positive miRNA detection/prediction. It might be due to the tissue-specific expression profile of many miRNAs. For example, hvu-miR156 is the second highest expressed miRNA in Pallas, whereas the same miRNA in Clipper is only ranked 14. In addition, in Pallas, hvu-miR156 is 0 order of magnitude from the highest expressed hvu-miR168, whereas in Clipper, this miRNA is over two orders of magnitude from the highest expressed hvu-miR167d (Supplementary Table S8). Both the highest expressed hvu-miR168 in Pallas and the highest expressed hvu-miR167d in Clipper have roughly the same scale in expression level (Supplementary Table S8). This suggests that miR156 might be highly shoot specific. Interestingly, the two highly expressed novel miRNAs (hvu-miRX21 and hvu-miRX22) in Pallas, which have not been detected in any other species so far, are not detected in Clipper either. Another highly expressed miRNA that is absent in Clipper is miR827, which is strongly up-regulated under deficient P (Supplementary Table S8) and might, therefore, not be in Clipper barley that grew under a normal (P) nutrition condition.

tsRNAs were the second most abundant sRNA class in both P treatments, accounting for 22.7% of the total sRNAs in P-deficient treatment and 19% in P-sufficient treatment. The high abundance of tsRNAs has been the case in epimastigotes and trypomastigotes, which accounts for 30 and 40% of the total sRNAs, respectively.[50] To our surprise, one tsRNA derived from the chloroplast-encoded tRNA-His(GTG) gene is the most abundant sRNA of all the sRNAs sequenced in both P treatments. Recent studies have shown that tsRNAs are Dicer dependent, can be incorporated into the RISC and direct Ago-mediated cleavage.[4,50,52,53] These suggest that tsRNAs can function in an miRNA-like pathway.[5,54] This would then imply that tsRNAs could play a certain role in the cells. Indeed, a tsRNA (tRF-1001) from human prostate cancer cells was recently demonstrated to be required for cell viability through the siRNA/miRNA pathway.[4]

Although tsRNAs are processed by Dicer-like endonucleases and associated with argonautes as miRNAs, they are derived from RNA polymerase III-catalyzed transcripts. In contrast, miRNAs are derived from RNA polymerase II transcripts. In addition, the sizes of tsRNAs are generally larger than those of miRNAs. In bacteria, fungi, plants and animals, the size of tsRNAs ranges from 30 to 35 nt.[4] Furthermore, tRNA genes are mostly cleaved at or around the anti-codon loop in both yeast and humans.[55,56] However, in barley, we found that the predominant size of tsRNAs was 20 nt, with the cleavage sites mainly occurring at the very 5′ ends of tRNA transcripts. This observation indicates that the biogenesis of tsRNAs in barley may adopt a mechanism different from tsRNAs in other organisms.

Chloroplast-derived sRNAs are a novel class of sRNAs. A large number of csRNAs have been identified in various species.[32,57–60] CsRNAs have been proposed to protect RNA from degradation.[61] However, the biogenesis mechanism of these csRNAs is unclear. It is believed that normal RNA degradation processes and the miRNA/siRNA pathway may both be involved. RNA degradation uses ribonucleases to cleave RNA at exposed sites. However, the main enzyme responsible for initial cleavage in the course of RNA degradation has not been identified in plant chloroplasts.[62] The miRNA/siRNA pathway adopts a specific Dicer ribonuclease to initially generate hairpin precursors, resulting in 20–24 nt short duplexes. However, this process may not be accomplished in the chloroplast *per se*, but in the nucleus, because the Dicer ribonuclease is not present in the chloroplast.[62] This could explain why so far no miRNAs have been identified in the chloroplast. In the case of the chloroplast-derived tsRNAs, which are the most abundant csRNAs in barley, their generation may involve RNase P, an endoribonuclease essential for processing polycistronic precursor tRNAs into mature tRNA 5′ ends.[63] In addition, other tRNA-specific nucleases, such as colicin E5, colicin D, prrC, ranpirnase or onconase and angiogenin, could be involved.[4] However, the involvement of such enzymes

cannot explain the preferential cleavage sites found in the tsRNAs.

P affects the expression of csRNAs (Table 1). This suggests that P may be involved in degradative changes in the chloroplasts. Previous studies have shown that P deficiency decreases the chlorophyll content to levels below what is required for chloroplast stability and the ability of the chloroplast to synthesize ATP.[64] This implies that csRNAs may result from a lack of energy available for photosynthesis and cellular respiration. However, we must point out that it cannot be determined, if csRNAs are derived from the chloroplast genome or derived from chloroplast-derived sequences inserted in the nuclear genome. Therefore, the data for csRNAs identified in this study may not truly reflect the real situation in the plants.

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Supplementary data: Supplementary Data are available at www.dnaresearch.oxfordjournals.org.

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