Pi-starvation induced transcriptional changes in barley revealed by a comprehensive RNA-Seq and degradome analyses

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Abstract

Background: Small RNAs (sRNAs) are 20–30 nt regulatory elements which are responsible for plant development regulation and participate in many plant stress responses. Insufficient inorganic phosphate (Pi) concentration triggers plant responses to balance the internal Pi level.

Results: In this study, we describe Pi-starvation-responsive small RNAs and transcriptome changes in barley (*Hordeum vulgare* L.) using Next-Generation Sequencing (NGS) RNA-Seq data derived from three different types of NGS libraries: (i) small RNAs, (ii) degraded RNAs, and (iii) functional mRNAs. We find that differentially and significantly expressed miRNAs (DEMs, p -value < 0.05) are represented by 162 (44.88 % of total differentially expressed small RNAs) molecules in shoot and 138 (7.14 %) in root; mainly various miR399 and miR827 isomiRs. The remaining small RNAs (i.e., those without perfect match to reference sequences deposited in miRBase) are considered as differentially expressed other sRNAs (DESSs, p -value Bonferroni correction < 0.05). In roots, a more abundant and diverse set of other sRNAs (DESSs, 1796 unique sequences, 0.13 % from total unique reads obtained under low-Pi) contributes more to the compensation of low-Pi stress than that in shoots (DESSs, 199 unique sequences, 0.01 %). More than 80 % of differentially expressed other sRNAs are up-regulated in both organs. Additionally, in barley shoots, up-regulation of small RNAs is accompanied by strong induction of two nucleases (S1/P1 endonuclease and 3'-5' exonuclease). This suggests that most small RNAs may be generated upon endonucleolytic cleavage to increase the internal Pi pool. Transcriptomic profiling of Pi-starved barley shoots identifies 98 differentially expressed genes (DEGs). A majority of the DEGs possess characteristic Pi-responsive *cis*-regulatory elements (P1BS and/or PHO element), located mostly in the proximal promoter regions. GO analysis shows that the discovered DEGs primarily alter plant defense, plant stress response, nutrient mobilization, or pathways involved in the gathering and recycling of phosphorus from organic pools.

Conclusions: Our results provide comprehensive data to demonstrate complex responses at the RNA level in barley to maintain Pi homeostasis and indicate that barley adapts to Pi-starvation through elicitation of RNA degradation. Novel P-responsive genes were selected as putative candidates to overcome low-Pi stress in barley plants.

Background

Barley (*Hordeum vulgare* L.) is one of the most commonly cultivated crop plants worldwide. It is a diploid plant with a low chromosome number ($n = 7$) and large genome size (haploid genome size of ~5.3 Gbp). In recent years, many resources essential to barley genomic studies have been developed, including a barley genome assembly in Ensembl Plants [1], a large number of expressed sequence tags (ESTs) [2], DNA markers, and useful techniques for stable or transient transformation of barley [3]. The simplicity of cross-breeding and cultivation in a wide range of climatic conditions makes barley a model crop plant in the study of desirable agronomic traits [4]. Studies on the responses of barley to abiotic stresses can help to better its cultivation in variable and adverse conditions. Environmental stressors cause crop damage

and reduction of yields, which result in financial losses for agricultural businesses. In plants, abiotic stresses trigger specific stress-induced molecular pathways that often involve different classes of small RNAs (sRNAs) [5–7].

Small RNAs (sRNA) are non-translating into protein class of RNA (20–30 nt) [8]. Best known are siRNA (small interfering RNAs) and miRNA (microRNAs, 18–25 nt) - a class of double-stranded RNA which may target chromatin or transcripts to regulate both the genome and transcriptome [9, 10]. Plant small RNAs tend to bind to Argonaute (AGO) family proteins to form either RNA-induced silencing complexes (RISC) for post-transcriptional gene silencing (PTGS) [11] or RNA-induced initiation of transcriptional silencing (RITS) complex for transcriptional gene silencing [12]. Recently, many studies have emerged about various sRNA types, biogenesis, targets, and functions [13–15]. Based on the biogenesis pathway, small RNAs have been classified into miRNAs, siRNAs, phasiRNA and tRFs (tRNA-derived RNA fragments) [16]. Among them, miRNAs and siRNAs are the most extensively studied sRNAs in plants.

Plant *MIR* genes represent independent transcriptional units, which are transcribed by RNA polymerase II (RNA Pol II). Primary transcripts (pri-miRNAs) mature in a two-step process in the cell nucleus [17]: Firstly, pri-microRNAs are diced out by the DCL1 (DICER-LIKE 1) protein from a stem-loop precursors [18]. The next step of DCL1 protein action leads to the generation of a double-stranded molecule composed of a guide miRNA strand and the passenger miRNA* (star) strand (called the miRNA/miRNA* duplex). Different DCL family members produce miRNA molecules of different lengths; however, the majority of plant miRNAs are 21 nucleotides in length [19]. The miRNA is assembled together with AGO1 (ARGONAUTE 1), in order to create RISC in the cytoplasm which is responsible for mRNA slicing. The cleavage position is precisely determined and occurs in the target mRNA between nucleotides complementary to the 10th and 11th nucleotides of the related miRNA, counting from the miRNA's 5'-end [20]. Ultimately, target mRNA recognized by the specific miRNA molecule is degraded by 5'-to-3' exonucleases and the overall pool of valid mRNA transcripts is decreased [21]. Such a mechanism exists in plants to modulate the expression levels of crucial stress-responsive genes [22].

In plants, there are many types of siRNAs, including (i) nat-siRNAs (natural-antisense siRNAs), which are produced from overlapping regions of natural sense–antisense mRNA pairs; (ii) ta-siRNAs (trans-acting siRNAs), processed from non-coding RNA precursors; and (iii) ra-siRNAs (repeat-associated siRNAs), generated from transposable and repetitive elements to mediate further steps of RNAi [9, 23]. tRFs may be produced after cleavage of tRNA ends (to generate 5'-tRF and 3'-tRF) by RNase T2 [24], as well as DCL (DICER-LIKE) processing in plants [25]. Both miRNAs and siRNAs mediate RNA interference (RNAi) in plants, but there are subtle differences between them. As an endogenous molecule miRNA is diced-out from microRNA precursor folded in stem-loop structure [26], while siRNA is a double-stranded RNA derived from the host genome or directly from viruses or transgenes [27].

The expression of sRNAs changes in response to environmental factors [7, 28] or viral infection [29–31]. Mentioned above classes of sRNAs appear to play important roles in plant growth, development regulation, and adaptation to various stresses. In barley, miRNAs have been shown to (i) mediate

tolerance to heat stress [32], (ii) confer drought tolerance [33], (iii) regulate low-potassium tolerance [34], (iv) respond to aluminum stress [35], and (v) maintain inorganic phosphate (Pi) homeostasis [36]. On the other hand, siRNAs mostly function as defenders of genome integrity in response to foreign nucleic acids [37]. The *TAS3* gene expresses ta-siRNAs, which may negatively regulate auxin signaling by targeting *AUXIN RESPONSE FACTOR 3* (ARF3) transcripts [38] and moderate floral architecture in response to drought stress in *Arabidopsis thaliana* L. [39]. The TAS-ARF pathway has been shown to be involved either in the development process of maize (*Zea mays* L.) [40] or regulating lateral root growth in *Arabidopsis* [41]. In addition, tRNA-derived small RNAs have been shown to accumulate in *Arabidopsis* roots under Pi-starvation [42], while rhizobial tRFs can regulate nodule formation in soybean (*Glycine max* L.) [13].

Changes in soil nutrient concentrations lead to aberrations in the set of sRNAs, with respect to the prevailing severe environmental conditions [6]. One of the most important macronutrients, which is indispensable for proper plant growth, is phosphorus (P) [43, 44]. P is a component of DNA, RNA, phospholipids, and ATP, and is involved in several biochemical processes such as protein phosphorylation, energy storage and transfer, and regulation of protein synthesis [45]. From soil matrices, P is acquired by the root system in the form of inorganic phosphate (Pi) ions. Insufficient Pi supply leads to barley growth inhibition [46, 47]. Plant transcriptome response to Pi-starvation involves protein coding genes, sRNAs, and long non-coding RNAs that form regulatory feedback loops. The most widely studied molecules in this context—miRNA399 molecules—are up-regulated in barley shoots and roots under low-Pi conditions [36]. MiRNA399 targets the 5'-UTR of the barley *PHO2* (*PHOSPHATE 2*) transcripts [48], encoding an ubiquitin-conjugating E2 enzyme (UBC24), a negative regulator of Pi uptake and root-to-shoot translocation. *PHO2* is involved in ubiquitination of *PHOSPHATE TRANSPORTER 1* (PHT1) family [49] and *PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR 1* (PHF1) [49]. Transgenic *Arabidopsis thaliana* plants overexpressing miR399 accumulate excessive Pi in shoots and display Pi over-accumulation toxic symptoms. Likewise, such a phenotype has been reported for the *pho2* loss-of-function *Arabidopsis* mutant [50, 51]. Thus, plants have developed a strategy to regulate the level of miR399 in the cytoplasm. The non-coding RNA molecule, *IPS1* (INDUCED BY PHOSPHATE STARVATION 1), has been shown to be highly expressed in plants exposed to Pi-starvation [52–54]. *IPS1* is a non-cleavable miR399 target which inhibits miR399-mediated down-regulation of *PHO2* mRNA by target mimicry [54]. Thus, the RNAi effect of miRNA activity may be counterbalanced by other RNAs, in a stress-dependent manner.

Deep sequencing of sRNAs has uncovered up-regulation of miRNAs like miR156, miR778, miR827, and miR2111, and down-regulation of miR169, miR395, and miR398 in *Arabidopsis* plants upon Pi deprivation [42, 55]. In rice (*Oryza sativa* L.), Pi-starvation induced the expression level of miR827 molecules, which dysregulate the transcript level of two genes encoding the SPX-MFS (named after proteins *SYG1*/*PHO81*/*XPR1* and the protein domain *Major Facility Superfamily*) protein family members SPX-MFS1 and SPX-MFS2 [56, 57]. These two SPX-MFS membrane transporters mediate Pi transport and control Pi homeostasis in shoot [58]. In *Arabidopsis*, the level of mature miR778 was up-regulated in shoots and roots in low-Pi conditions, while its target gene expression *SUVH6* (*SU(VAR)3-9 HOMOLOG 6*)

was accordingly reduced [59]. The *SUVH6* gene encodes a histone H3 lysine 9 (H3K9) methyltransferase, which may enable plants to adapt to environmental conditions by changing their chromatin structure [60]. miR2111 functions as an activator of rhizobial nodulation, which is strictly correlated with the balanced assimilation of nitrogen (N) and P in plants [61, 62]. However, there is still a gap in understanding how Pi-starvation affects the quantity and quality of sRNAs distributed in barley shoots and roots. What kind of sRNAs are preferentially induced? What is the role of sRNAs in responding to Pi-starvation? What are the mRNA targets recognized by those sRNAs in barley?

In this paper, we analyzed changes in the expression levels of RNAs in barley growing under Pi-starvation, as compared to control/Pi sufficient conditions. Our results support the hypothesis that Pi-starvation triggers underlying molecular mechanisms and the expression level of key genes involved in maintaining proper barley growth and development. Combined deep sequencing data (sRNAs, degradome and mRNAs) reveals the widespread importance of low-Pi-dependent miRNAs and genes representing various biological pathways. Using degradome analysis, we identified mRNAs targeted by sRNAs identified in this study. Among these sRNAs, only a small fraction maps perfectly to miRNA sequences deposited in miRBase. Our degradome data show that most sRNAs produced upon Pi-starvation are not involved in gene silencing. In addition, we performed transcriptome analysis of the protein-coding gene expression in barley shoots upon Pi-starvation. Subsequent analyses were performed (GO analysis, chromosomal mapping, and Pi-responsive motifs localization) to characterize specific stress responses in barley plants to accomplish Pi homeostasis.

Results

Barley plants display low-Pi symptoms at the morphological and molecular levels

Severe low-Pi responses were induced in the barley plant line Rolap grown in the soil containing 8 mg P/kg. P undernourishment caused over 2-fold reduction of plant shoot biomass (**Fig. 1A**). Shoot fresh weight of plants at 23rd day post-sowing (dps) was significantly reduced, in comparison with control plants, with average mass 8.8 g for stressed plants and 18.5 g for plants growing under Pi-sufficient conditions ($p = 0.001$) (**Fig. 1B**). We observed a significantly decreased concentration of Pi ions, with only 0.48 μmol Pi per g of fresh root weight (FW) and 4.2 μmol Pi per g of shoot FW, when compared with the control plants having 3.84 ($p = 0.0056$) and 24.35 μmol Pi/g FW ($p = 0.0001$), respectively (**Fig. 1C**). To examine the induction of changes at a molecular level by low-Pi stress in barley plants, we measured the absolute gene expression of the low-Pi-responsive marker gene *IPS1*. The barley *IPS1* gene is highly expressed under Pi-deficient conditions in the plant line Rolap. At the tillering stage (23 dps), we detected 4191 copies of *IPS1* RNA for low-Pi treated roots, normalized per 1000 copies of *ADP-RIBOSYLATION FACTOR 1-LIKE* (*ARF1*) reference gene, in comparison to the control plants, with only 58 copies of *IPS1* RNA ($p = 0.00006$) (**Additional file 1**). Taking validated plant material, we performed tripartite deep-sequencing analysis to: (i) identify Pi-responsive sRNAs, (ii) elucidate changes in the barley transcriptome upon Pi starvation, and (iii) identify mRNA targets for Pi-responsive sRNAs through degradome sequencing (**Fig. 2**).

Barley plants express an organ-specific set of microRNAs in response to low-Pi conditions

We performed small RNA deep-sequencing to find out which small RNAs are up- or down-regulated by Pi starvation in barley shoots and roots. The average of 30.4 mln reads for roots and 25.2 mln reads for shoots were generated in 50 nt single-read Illumina sequencing (**Additional file 2**). After adapter and quality trimming, we mapped reads to the miRBase Sequence Database (release 22) to annotate miRNA-derived sequences [63]. A set of parameters were used to define the pool of differentially expressed miRNAs: (i) no mismatches with the reference sequences in the miRBase were allowed; (ii) different types of miRNA sequences were permitted, whether they were annotated as precursor, mature, or isomiR; (iii) miRNA sequences were named accordingly to the name of the assigned reference miRNA; and (iv) significance of fold change (p -value < 0.05) was additionally verified using a restricted Bonferroni p -value test (**Fig. 2**).

We found 138 and 162 differentially expressed miRNAs (DEMs) annotated to the miRBase (p -value < 0.05) in barley roots and shoots, respectively. Only 25 DEMs were expressed in both examined barley organs (**Additional file 3**). However, restricted Bonferroni p -value correction narrowed down set of miRNAs to 15 in shoots and 13 in roots (**Fig. 3A, left panel**). We focused further only on those that passed the Bonferroni test (**Fig. 3A, right panel**). In both organs, most of the DEMs were significantly up-regulated. Interestingly, out of 15 miRNA, only miRNA166d was down-regulated in shoot under low-Pi ($\log_2$ (fold change) = -1.18). In our previous work, we showed that miRNA166 is expressed in barley during different developmental stages reaching the highest level in 2-week-old plants [64]. miRNA166 plays an important role in plant development, including root and leaf patterning, by targeting mRNA encoding HOMEODOMAIN LEUCINE-ZIPPER CLASS III (HD-ZIP III) transcription factors [65]. Similarly, only miRNA319b out of 13 DEMs was down-regulated in low-Pi treated roots ($\log_2$ (fold change) = -1.28). In a previous study, we presented data that Arabidopsis miR319 is a multi-stress responsiveness miRNA [22]. For example, *MIR319b* gene expression was down-regulated in response to drought, heat, and salinity, but up-regulated in response to copper and sulfur deficiency stresses [22].

Uncharacterized miRNAs are highly induced by low-Pi stress in barley roots

A specific set of miRNAs was expressed in barley shoot or root under low-Pi (**Additional file 3**). In shoot, only two miRNA families, miRNA399 and miRNA827, were induced, while in root we observed a more diverse response (**Fig. 3A, right panel**). Apart from miRNA399/miRNA827 induction, we found the following additional miRNA to be up-regulated in root: two miRNA5083 (id = 3, and id = 4), miRNA1511 (id = 6), two miRNA9779 (id = 16, and id = 17), two miRNA156 (id = 65, and id = 69), and miRNA5072 (id = 118). Among these eight miRNAs, only miR156 has been reported before as Pi-responsive in Arabidopsis [42, 55]. The miR156 isomiRs were also found dysregulated in shoot, but none of them pass the Bonferroni test. Our results suggest that there is a more complex response to low-Pi stress regarding miRNA expression in roots than in shoots, where the miRNA action is directed to control the transcript level of either *PHO2*, *SPX-MFS1*, or *SPX-MFS2* by just two miRNA families.

In order to determine the potential cleavage activity of miRNAs identified in root we performed degradome analysis. None of these eight miRNAs were found to recognize a specific target from the pool of barley mRNAs. Our result raises the hypothesis that plants may release Pi from the pool of stress-responsive small RNAs. In addition, most of them match to the pre-miRNAs, not mature molecules (**Fig. 3A, right panel**).

NGS (Next Generation Sequencing) data were validated by complex analysis of mature miR827 (from 3' arm) in all samples taken for deep sequencing. The absolute expression level of miR827 is significantly up-regulated in both shoots and roots under a low-Pi regime (**Fig. 3B**). The $\log_2$ fold change of miR827 molecules defined by deep-sequencing in shoot (id: 2073) was found on the same level in root (id: 114), $\log_2(\text{fc}) = 3.05$ and 3.01 , respectively (**Fig. 3A**). The ddPCR results were consistent with NGS data showing up-regulation of mature miR827 molecule in both tested organs (**Fig. 3B**). These data were confirmed by northern blot hybridization (**Fig. 3C**) and degradome analysis (**Fig. 3D**). The results showed that *SPX-MFS1* transcripts have been recognized and cleaved by Ago protein associated with miR827 in barley ($p = 0.014$) (**Fig. 3D**).

Different classes of small RNAs in barley accumulate in an organ-specific manner under low-Pi regime

The small RNA reads which did not map to miRBase were mapped to particular classes of barley cDNAs derived from the Ensembl Plants database (release 40), both separately and to all of them (**Fig. 2**). All sequences mapped to barley cDNAs are listed in **Additional file 3**. We found that small RNAs, other than miRs, differentially expressed (DESS) in barley under Pi starvation were represented by 199 unique sequences (0.01 % of all unique reads from barley shoots (**Additional file 4**) and by 1796 (0.13 %) unique reads from roots (**Fig. 4A, Additional file 5**).

We analyzed whether different lengths (taking sequences from 18 to 25 nt in length) and classes of small RNAs contributed to either root or shoot response to low-Pi conditions. In roots, the length distribution of DESS remained balanced, from 10.91 % for the representation of 24 nt sequences to 15.26 % for the 18 nt sequences, which were the most abundant (including 274 DESS) (**Fig. 4B**). In shoots, the representations of DES lengths fluctuated more than in roots. The 19 nt sequences were the most visible (21.11 %), while three representations did not score more than 10%: the 22 nt (9.55 %), 23 nt (8.54 %), and 25 nt (3.52 %) sequences (**Fig. 4B, Additional file 6**).

In roots, 1070 unique reads were mapped to cDNA sequences annotated in the Ensembl Plants databases (non-translating, protein-coding, pseudogenes, rRNA, snoRNA, snRNA, sRP-RNA, tRNA), while 726 unique sequences remained without match. The DESS obtained from low-Pi roots were mostly annotated to protein-coding mRNAs (38.54 %), rRNAs (34.17 %), and non-translating RNAs (19.49 %). Below 5 % of overall DESS, we found a number of remaining cDNA classes, such as snoRNAs (2.49 %), tRNAs (2.47 %), SRP-RNAs (1.17 %), snRNAs (0.95 %), and pseudogenes (0.65 %). While in shoot, we found 199 DESS under the low-Pi regime. Altogether, 116 out of 199 differentially expressed small RNAs (DESS) were annotated to the barley Ensembl Plants database, where 83 sequences remained without match

(**Additional file 4**). In the case of shoot samples, 85 % of annotated DESs represented only protein-coding mRNAs (47.87 %) and non-translating RNAs (36.49 %) (**Fig. 4A**, right panel; **Additional file 7**). We did not find any DESs annotated to the snRNAs, SRP-RNAs, or tRNAs from barley shoot upon low-Pi. In addition, total numbers of 166 DESs (83 %) in shoots and 1560 DESs (87 %) in roots were significantly up-regulated after exposure to low-Pi stress (**Additional file 4**, **Additional file 5**, **Fig. 7**).

Among the unannotated reads of sRNAs in roots, the highest fold change was observed for a 19 nt 5'-ACCTACTCGACCTCGGCCG-3' molecule ($\log_2(\text{fold change}) = 8.02$, induction) and a 22 nt 5'-CTAATACCGGATACGCGAACCG-3' molecule (-5.87 , repression). The BLAST (Basic Local Alignment Search Tool) analysis of first (19 nt) molecule showed a perfect match to either the intergenic region of barley chromosome no. 5, soil bacteria (*mesorhizobium*), or *Linum usitatissimum* L., while the second molecule (22 nt) mapped to RNA encodes 16S rRNA. Furthermore, in roots, the most abundant small RNA was a 25 nt 5'-ACCGACCTACTCGACCCTTCGGCCG-3' molecule (15847.7 and 65590.5 mean of normalized counts in barley root in control and low-Pi conditions, $\log_2(\text{fc}) = 2.82$). This small RNA matched several barley loci encoding SSU (small subunit) rRNAs (**Additional file 5**).

In our results from low-Pi treated shoot samples, the highest fold change was represented by a 24 nt 5'-AAGATTGGTTGGTTGGTTGGGTCT-3' molecule ($\log_2(\text{fc}) = 8.72$, induction). This 24 nt molecule is a part of transcript encoding a putative pentatricopeptide repeat (PPR) protein. The PPR protein family facilitates the processing, splicing, editing, stability, and translation of RNAs in plants [66]. The most abundant small RNA was a 19 nt 5'-GGGCCTGTAGCTCAGAGGA-3' molecule (9471.5 and 49914.1 normalized mean counts in barley shoot in control and low-Pi, respectively, $\log_2(\text{fc}) = 2.45$). This sRNA was mapped to the barley genomic loci (EPIHVUG00000039813), which encodes arginyl-tRNA (trnR-ACG) and a cDNA encoding uncharacterized protein (HORVU2Hr1G084630) which is likely involved in carbon fixation. Interestingly, the pool of DESs was selective, considering organ-specific expression change, providing only three unique sequences that were significantly changed in both barley organs under low-Pi regime (**Fig. 4A**, left panel). These molecules were: (i) 20 nt 5'-AGTAGAGGTTCGCGAGAGAGC-3' ($\log_2(\text{fc}) = 2.01$ in root and 1.16 in shoot, respectively) annotated to the 26S rRNAs, (ii) 24 nt 5'-ATTCTCCGCGTCGGATACCTGAGA-3' (3.69 in root and 2.07 in shoot) encoding the barley MYB21 transcription factor, and (iii) 21 nt 5'-TGCCAAAGGAGAACTGCCCTG-3' (4.64 in root and 6.27 in shoot) mapped to the intergenic region of barley chromosome no. 3 (**Additional file 4**).

The results obtained in this study show again that barley roots exhibit a more diverse pool of Pi-responsive small RNAs which may trigger developmental adaptation of the root to Pi-starvation. Additionally, 613 rRNA-derived sRNAs are up-regulated, whereas 176 rRNA-derived sRNAs are down-regulated in barley roots (**Additional file 5**). We believe that such sRNA may be further processed, serving as a Pi source to compensate Pi deficiency.

Identification of barley genes responsive to Pi-starvation

Since we observed, that most of the other sRNAs in shoot were derived from either protein-coding mRNAs or non-translating RNAs, we checked whether this observation is correlated with gene expression changes of polyadenylated RNAs in barley shoot under Pi-starvation. Among 98 of identified DEGs, the transcripts of 56 annotated loci were significantly up-regulated, while those derived from 42 loci were down-regulated in Pi-starved barley shoots (**Additional file 8**). Repressed loci were found to be preferentially located at barley chromosome no. 2, while induced loci were found mostly at barley chromosomes no. 3 and 5 (**Fig. 5B**).

The highest enrichment of DEGs was found in the GO terms, either (i) belonging to the cellular components of the chloroplasts; (ii) showing catalytic activity, either ion or metal binding properties; and (iii) involved in the various biological and metabolic processes related to stress response and plant defense (**Fig. 5A**). A major set of up-regulated DEGs represented genes involved in the Pi signaling. Among them, we found genes encoding: *IPS1* ($\log_2(\text{fc}) = 5.89$) [54], inorganic pyrophosphatase (PPase, 4.01) [67], *SPX*-domain containing protein 5 (*SPX5*, 3.44) [68], phosphate transporter *PHOSPHATE 1-3* (*PHO1-3*, 2.97) [69], *SPX-MFS2* (2.79) [56], *haloacid dehalogenase-like hydrolase* (*HAD1*, 1.95), [70] and five different purple acid phosphatases (PAPs) (**Fig. 5C; Additional file 8**) [71]. Interestingly, four genes were induced to a higher extent than the low-Pi stress marker, *IPS1* gene. These genes encode ferredoxin (*FD1*, $\log_2(\text{fc}) = 14.20$), mitochondrial-processing peptidase (13.35), chlorophyll a/b binding protein (8.90), and alpha-amylase (7.30), and are engaged in photosynthesis, redox reactions, reactive oxygen species (ROS) homeostasis, and co-ordinated mobilization of nutrients. Chloroplasts and mitochondria are the organelles with the highest Pi requirements. Strong *FD1* gene up-regulation most likely reflects the accumulation of reduced ferredoxin in chloroplasts. Low-Pi lowers the capacity to process incoming light and enhances starch accumulation in chloroplasts, thereby leading to photoinhibition [72, 73]. Within the category of genes that were significantly down-regulated, most of them were related to stress and defense responses (**Additional file 8**); for instance, uncharacterized protein (HORVU2Hr1G030090, -6.50), oxalate oxidase (-4.41) [74], beta-sesquiphellandrene synthase (-3.41), glutamate carboxypeptidase (-3.17), chalcone synthase (-3.05) [75], or caleosin-like protein (-2.95) (**Fig. 5C**). Only two repressed genes are known to be directly involved in Pi signaling and metabolism, *SPX-MFS1* (-2.58), targeted by miR827 [57] and probable inactive purple acid phosphatase (-1.75). Additionally, two genes encoding laccases (*LAC19-like*, **Additional file 8**), cell wall-localized multi-copper oxidases, were significantly down-regulated (-2.10 and -2.44) in our mRNA RNA-Seq data. Laccases are involved in copper homeostasis (**Fig. 7**) and lignin biosynthesis, and have been shown to be targeted by miR397 in maize [76] and Arabidopsis [77]. Furthermore, key genes encoding proteins involved in the nitrate and phosphate cross-talk were affected by low-Pi conditions in barley shoots, such as *NIGT1* (*NITRATE-INDUCIBLE, GARP-TYPE TRANSCRIPTIONAL REPRESSOR 1*) transcription factor (3.80) [78, 79] and nitrite reductase (1.98), as well as high-affinity nitrate transporter *NRT2.1* (*NITRATE TRANSPORTER 2.1*) (-2.60) [80].

Absolute quantification of a few selected transcripts was performed to validate RNA-Seq data obtained in this study. Two genes which were highly induced (encoding endonuclease S1/P1 and 3'-5'-exonuclease) and two which were severely repressed (encoding oxalate oxidases) under the low-Pi regime were taken

for ddPCR (droplet digital PCR) analysis (**Fig. 6A**). We confirmed statistically significant changes ($p < 0.05$) in normalized copy number (per 1000 copies of the *ARF1* reference gene) of all genes taken for analysis.

Pi-responsive motifs found in the promoters of DEGs

In general, genes that are affected by Pi status possess characteristic *cis*-regulatory elements within either promoter or 5'-UTR regions [81]. Previously, we have shown the importance of the P1BS motif (PHR1 binding sequence, consensus G_nATATnC, [82]) and P-responsive PHO elements (consensus ATGCCAT, [83]) in the expression efficiency of the barley *PHO2* gene [48]. Both motifs may bind PHR-like (PHOSPHATE STARVATION RESPONSE) transcription factors (TFs) and act as activators or repressors of downstream gene expression in a Pi-dependent manner [84]. Likewise, we hypothesized that regulatory regions of the identified DEGs had Pi-responsive motifs, which may be bound by PHR TFs, causing gene expression dysregulation. To confirm this hypothesis, we analyzed DNA sequences from the 2000 bp region upstream of the predicted transcription start sites from all 98 DEGs (**Additional file 9**). In the next step, promoter data were directly screened for P1BS and P-responsive PHO element consensus sequences by multiple promoter analysis using the PlantPAN3.0 tool [85]. We confirmed the presence of Pi-dependent motif in 55 out of 98 DEGs promoters. An *in silico* approach detected 46 DEGs having at least one P1BS motif (**Additional file 10**) and 17 DEGs with at least one P-responsive PHO element (**Fig. 6B, Additional file 11**). The most over-represented motifs were found in the promoters of genes encoding sulfoquinovosyl transferase SQD2-like ($\log_2(\text{fc}) = 3.74$) [86], phosphoenolpyruvate carboxylase 1-like ($\log_2(\text{fc}) = 1.73$) [87], and pyridoxal phosphate-dependent transferase ($\log_2(\text{fc}) = -2.61$) [88]. Each of the genes harbor three P1BSs and one P-responsive PHO element, as well (**Additional file 8**).

Degradome profiling describes post-transcriptional regulatory network of identified DEMs

After identification of (i) differentially expressed miRNAs (DEMs), (ii) other sRNAs (DESS), and (iii) mRNAs (DEGs), we used this comprehensive data together with cDNAs annotated in the Ensembl Plants database to identify the sRNAs directly involved in RNA degradation. The DESSs were also examined, because we assumed that there may have been putative miRNAs that were not mapped to the miRbase, due to restricted query settings allowing no mismatch. Molecules which exhibited a single mismatch (or more) may still function as miRNA in barley. Degradome libraries were carried out for root, as well as for shoot, and sequenced using an Illumina System. The received data were analyzed using two independent *in silico* approaches (**Fig. 2**). At times, the different algorithms used elicited different miRNA targets; however, the general degradome pattern was equivalent for both approaches (**Fig. 3A**, right panel, **Additional files 12-24**).

First, we searched for the potential target mRNAs for a broader set of differentially expressed miRNAs with significant fold change (p -value without Bonferroni correction), taking 162 DEMs from shoot and 138 DEMs from root, respectively (**Additional file 3**). A total of 1964 scores were obtained for shoot DEMs (1303 using the TargetSeek approach and 661 using PAREsnip2) (**Additional file 12, Additional file 16**),

while in root there were 1515 records (921 and 594, respectively) (**Additional file 14, Additional file 20, Additional file 21**). Accordingly, shoot examination proved the proper selection of miRNAs from all differentially expressed small RNAs, as all 661 records predicted by the PAREsnip2 approach were categorized with the best score (0). A majority of records corresponded to different miR399 and/or miR827 isomiRs and their known targets PHO2 or SPX-MFS1/SPX-MFS2, respectively. In shoot, the best scoring miRNA:mRNA match was found for mature miRNA399d (21 nt, id = 2066), which guides cleavage within the 5'-UTR of *PHO2* mRNA (isoform no. 3) in position 857 (TargetSeek: score = 0, MFE = -41.1, PAREsnip2: score = 0, MFE = -41.1) (**Table 1**). The miR5049e (21 nt) targets various isoforms of mRNA encoding ENT domain-containing proteins with high probability (TargetSeek: score = 1.5, MFE = -27.4), which may be involved in the plant defense response to fungal infection and anthocyanin biosynthesis [89, 90]. Moreover, we found two target mRNAs (HORVU3Hr1G094730, HORVU6Hr1G031450) encoding SPL-like TFs guided for cleavage by two miR156 isomiRs (id: 2053 and 2054) in barley shoot (**Additional file 16, Additional file 17**). The *SPL* genes are post-transcriptionally regulated by miR156 and control shoot branching in rice [91].

In contrast, in roots, where the pool of identified DEMs is more diverse, the PTGS regulatory network involves many TFs with various functional domains (**Additional file 14**). For instance, the 21 nt mature miR444b (id = 101) targets mRNA encoding MADS-box transcription factor 57 (HORVU6Hr1G073040, TargetSeek: score = 0, MFE = -40.1, PAREsnip2: score = 0, MFE = -40.1), which regulates long-distance nitrate transport and root elongation in rice [92] (**Table 1**). The well-known abiotic stress-responsive miR319c (21 nt) [6] was found to target various isoforms of GAMyb TF (HORVU3Hr1G079490, TargetSeek: score = 5, MFE = -34.6, PAREsnip2: score = 3, MFE = -35.4). The same predicted miR319 target has been previously found in RNA-Seq of Pi-starved tobacco plants [93]. Its targets, the plant GAMyb TFs, have been shown to activate gibberellin-responsive gene expression of α -amylase in barley [94, 95]. Our degradome data indicate the possible cleavage of various mRNA isoforms encoding nuclear transcription factor Y subunit A-4 (NFYA4, HORVU4Hr1G005670, TargetSeek: score = 3, MFE = -27.6, PAREsnip2: score = 2, MFE = -28.1) mediated by miR169d (20 nt, id = 84) (**Table 1**), what is consistent with previous studies on *Cunninghamia lanceolata* L. showing that NFYA4 mRNA is targeted by miR169d (authors used PAREsnip approach) [96]. The NFYA4 protein is involved in many abiotic stress responses and may regulate the timing of transition from vegetative to reproductive phase [97]. All predicted DEM targets for both organs are listed in **Additional files 13, 15, 17, and 21**.

Putative regulatory small RNAs identified in degradome data

Degradome profiling was performed to test whether any of the sequences from the 1796 DESs found in roots or 199 DESs found in shoots contribute to the complexity of gene regulation during low-Pi stress. A total of 759 records (245 using the TargetSeek approach and 514 using PAREsnip2) were found in the degradome profiles matching root DESs (**Additional file 15, Additional file 22, Additional file 23**) and 160 records (87 and 73, respectively) matching shoot DESs (**Additional file 13, Additional file 18, Additional file 19**). Taking only either the most up-regulated or the most down-regulated sRNAs for degradome screening, we found six promising target genes in shoot and five in root (**Table 2**). For example, in roots,

the highly up-regulated 20 nt sequence 5'-ACCGACCTACTTGACCCTTC-3' ($\log_2(\text{fc}) = 6.46$, id = 348) binds to the 3'-UTR region of the MYB44 TF's mRNA and guides/promotes cleavage in the 1037 position (PAREsnip2: score = 4; MFE = -33.3) (**Table 2**). RNA-Seq data for potato (*Solanum tuberosum* L.) proved that expression of the *MYB44* gene is highly downregulated under low-Pi in roots [98], which may be the result of miRNA-guided PTGS. Studies in potato have indicated that MYB44 TF may form a regulatory complex together with WRKY6 TF, which negatively regulates Pi transport by suppressing *PHO1* expression [98]. Other degradome records, among the most differentially expressed sRNAs, were found to target mRNAs of the V-ATPase assembly factor (VMA21-like) and three barley genomic loci encoding uncharacterized proteins (HORVU7Hr1G053570, HORVU1Hr1G027340, and HORVU0Hr1G023910) (**Table 2**). For example, the potential cleavage activity was predicted for 24 nt sequence 5'-AGAGGAAACTCTGGTGGAGGCTCG-3' ($\log_2(\text{fc}) = -3.58$, id = 463), which may target the mRNA encoding uncharacterized protein with unknown PTHR47188 domain (**Fig. 4C**).

Analogous degradome screening was done for shoot data. Among all identified DESs, we found that the most upregulated sequence, 24 nt 5'-AAGATTGGTTGGTTGGTTGGGTCT-3' ($\log_2(\text{fc}) = 8.72$, id = 2112), targets the 3'-UTR of mRNA encoding multiple organellar RNA editing factor 9 (MORF9, HORVU7Hr1G073170, TargetSeek: score = 16.5, MFE = -28.1) (**Table 2**). MORF9 proteins are required for RNA editing in plastid mRNAs, which may contribute to stress adaptation in plants [99, 100]. In both approaches, we found that the 21 nt sequence 5'-TGCCAAAGGAGAACTGCCCTG-3' (6.27, id = 2265) targeted the same isoform of *PHO2* mRNA (HORVU1Hr1G085570.3, TargetSeek: score = 6, MFE = -29.9, PAREsnip2: score = 3.5, MFE = -33.4). When we browsed the miRBase using this 21 nt sRNA as a query, we found high similarity to the osa-miR399a, exhibiting only one mismatch. Thus, we suspect that such sRNA may function as another miR399 isomiR in barley. Most dysregulated DESs were also found to target mRNAs encoding methyltransferase type 11 domain-containing protein (MT11), AAA-ATPase (At3g50940-like), lysine-specific demethylase 5A (LSD), or an uncharacterized protein with a predicted transmembrane domain (HORVU3Hr1G036970). The best scoring records were found for the 21 nt sequence 5'-TTAGATGACCATCAGCAAAC-3' (3.11, id = 2279), which targets mRNAs encoding SPX-MFS1 (PAREsnip2: score = 2.5, MFE = -27.5) and SPX-MFS2 (TargetSeek: score = 3.5, MFE = -26) (**Table 3**). This result suggests that such sRNA may exist as another miR827 isomiR. Moreover, this is consistent with the screening made for differentially expressed miRNAs, where miR827 targeted both SPX-MFS proteins, depending on the approach we used (**Fig. 3A**). In addition, the 18 nt sequence 5'-AATCGTCTTTACATCGGATG-3' (3.24, id = 2117) was found to target mRNA encoding glutaminyl-peptide cyclotransferase (HORVU7Hr1G003920, PAREsnip2: score = 2.0, MFE = -19.6) (**Fig. 4C**), which may be involved in plant defense reactions [101].

Some other interesting Pi-related targets which are recognized by DESs were found in our root degradome data, but the prediction scores were weaker than those in the examples described above. For instance: nitrate reductase (HORVU6Hr1G003300), high-affinity nitrate transporter-activating protein (HORVU5Hr1G115500), MYB-like TF (HORVU7Hr1G027370), and stress-induced TF NAC1 (HORVU5Hr1G111590) were found. Interestingly, among the 98 DEGs identified in this study, only two of

them (SPX-MFS1 and SPX-MFS2) were found as putative targets of miRNA guided activity. In addition, none of the DEGs gene IDs were found to match with any of the identified IDs classified for differentially expressed small RNAs. All predicted DESs targets are listed in **Additional files 14, 16, 19, and 23**.

Discussion

In this study, we used a tripartite approach (sRNA-Seq, mRNA RNA-Seq, and degradome-seq) to describe the set of small RNAs differentially expressed in barley roots and shoots under low-Pi stress. We detailed the sophisticated responses of barley shoots and roots involved in the maintaining of Pi homeostasis (**Fig. 6**). Integrated deep-sequencing data were used to describe organ-specific adaptations to low-Pi through either activation or repression of different classes of 18–25 nt small RNAs. Additionally, the mRNA-Seq analysis of low-Pi treated barley shoot was performed to analyze the correlation between shoot-derived small RNAs, annotated to either protein-coding mRNAs (47.87%) or non-translating RNAs (36.49%), and gene expression changes of polyadenylated RNAs. We identified a total of 28 differentially expressed miRNAs (Bonferroni tested *p*-value) annotated to miRBase (release 22) without mismatches and a total of 1989 differentially expressed other small RNAs (Bonferroni tested *p*-value).

In plants, a limited number of miRNA have been shown to be specifically and strongly induced by Pi limitation, including miRNA399 [102], miRNA778 [59], miRNA827 [55], and miRNA2111 [55, 103]. In this work, the majority of DEMs represent various miR399 and miR827 isomiRs in both tested organs. Our results are consistent with sRNA sequencing data published for Arabidopsis [42, 55] and *Nicotiana benthamiana* L. [93]. In both plant species, authors have shown that the number of various miR399 isomiRs was the most abundant in shoots and roots under low-Pi. Eight of the 15 DEMs (after Bonferroni *p*-value correction) we found in barley shoots belonged to the miR399 family. However, in root, miR399 was represented only by one DEM; the miR399b isomiR (**Fig. 3A, right panel**). Previously, our absolute copy number analysis of mature miR399 demonstrated that its normalized expression level is 4-fold downregulated in barley roots, as compared to in shoots, under a low-Pi regime [104]. The long-distance movement of signal molecules is known to be crucial for Pi recycling and allocation from root to shoot. The root system is responsible for Pi acquisition conducted by phosphate transporters belonging to PHT1 family, which saturate cell membranes during Pi deficiency [105]. The level of PHT1 proteins is negatively controlled by the PHO2, which is suppressed by miR399 (see model in **Fig. 7**) [106]. A high level of miR399 molecules was detected in Arabidopsis wild type rootstocks grafted with miR399-overexpressing scions [42, 107]. Thus, miR399 is involved in a plant's systemic response to low-Pi conditions and acts as a long-distance signal, moving from shoot to root to control Pi homeostasis [107]. In Arabidopsis, miR827 has been shown in multiple studies to target the 5'-UTR of the *NITROGEN LIMITATION ADAPTATION* (*NLA*) gene [108, 109]. In rice, the *OsNLA* mRNA has a 'degenerate' osa-miR827 potential cleavage site, that is why miR827 does not cleave the *OsNLA* transcript *in vivo* [56, 57, 110]. Likewise, we did not find *NLA* mRNAs to be targeted by any of the identified hvu-miR827 isomiRs in our barley degradome records. The *NLA* gene encodes an E3 ubiquitin-protein ligase with RING and SPX domains, which interacts with the PHO2 to prevent the excessive accumulation of Pi [111]. In roots, a more diverse set of miRNAs contributed to the compensation of low-Pi stress, compared to that in shoots. We found six up-regulated

miRNA molecules (DEMs) in roots mapped to pre-miRNAs, such as: miR9779, miR5083, miR9779, miR5083, miR1511 and miR5072. In addition, none of them was found in our degradome analysis. The differentially expressed other small RNAs in roots (DESS, 1796 molecules) were represented by 90 % of the total set of other sRNAs (DESS from both organs), annotated to all classes of cDNAs taken for analysis. Among the identified set of DESS, many putative miRNA-like molecules were predicted to target various mRNAs involved in plant adaptations to abiotic stresses, plant defense, and/or transcription (**Table 1**). Further analysis will be performed to experimentally validate the *in silico* predicted PTGS role of Pi-responsive small RNAs found in this study, as well.

In this paper, we detailed the particular shoot differentially expressed genes (DEGs) harboring Pi-responsive *cis*-regulatory elements, involving various molecular pathways and biological processes. These DEGs were mostly engaged in Pi mobilization and utilization upon Pi-starvation in barley shoots. Other sRNAs selected from shoots were much less abundant and represent sequences belonging mostly to non-translating and/or protein-coding mRNAs. None of the sRNAs mapped to the differentially expressed mRNAs found in the transcriptomic analysis, suggesting that they may inhibit gene expression through translational repression or may serve as a Pi source for developing plant organs. Plants are adapted to recycle nutrients from senescing organs. For example, class II RNases are involved in the degradation of housekeeping rRNAs before cell death occurs [112]. During senescence extracellular class I RNases were shown to degrade RNA during Pi-starvation in Arabidopsis as well [113]. In 2018, Ren *et al.* published RNA-Seq data describing the barley transcriptome under low-Pi stress [114]. The authors compared the transcriptomes of two barley genotypes with contrasting low-Pi stress tolerance. In roots, they observed 28 DEGs classified into the following functional groups: Pi transport, transcription, lipid metabolism, metabolism, and phosphorylation/dephosphorylation [114]. Likewise, our RNA-Seq data from barley shoot discovered the DEGs involved in all mentioned functional groups. In our shoot transcriptome analysis, we found the same four DEGs: (i) GLYCEROPHOSPHODIESTER PHOSPHODIESTERASE 1 (*GDPD1*) gene (HORVU3Hr1G079900, $\log_2(\text{fc}) = 5.78$), (ii) MONOGALACTOSYLDIACYLGLYCEROL SYNTHASE 2 gene (MGD2, HORVU4Hr1G044140, 5.27), (iii) *SPX5* (HORVU2Hr1G031400, 3.44), and (iv) *SPX1* (HORVU7Hr1G089910, 1.78). Furthermore, Ren *et al.* found three genes encoding purple acid phosphatases (PAPs) [114]; however, they appeared from different barley genome loci than the five PAPs we found in our study. It was shown that vacuolar and secreted PAPs are involved in Pi scavenging and remobilization during Pi-starvation and leaf senescence. Other related RNA-Seq data published for either wheat (*Triticum aestivum* L.) [115], rice [116], soybean [117], *Plantago major* L. [118], and maize [119] demonstrated similar molecular patterns to those in our study (**Additional file 8**). Based on our work we propose a model of barley adaptations to Pi-starvation (**Fig.7**). Interestingly, the presence of crucial Pi-responsive *cis*-regulatory elements within the promoter regions of more than 50 % of identified DEGs may indicate their essential and direct role in conditioning low-Pi tolerance (**Fig. 6B**). The most widely studied PHR TFs, such as PHR1 in Arabidopsis [82] and PHR2 in rice [120], bind to P1BS elements present in the promoter of a broad range of Pi-related genes. Moreover, the PHR protein family exhibits high functional redundancy and its protein members may co-operatively form a regulatory network to maintain Pi homeostasis in plants [84]. In our previous paper, we showed that,

within the 5'-UTR of the *PHO2* gene, there is another Pi-responsive motif called the PHO element in close proximity to the P1BS [48]. The PHO element can be bound by PHR-like transcription factors in barley plants, as well [48], and has been found in the promoters of many DEGs in independent Arabidopsis [121, 122] or soybean [117] studies.

The elevated abundance of sRNAs has been associated with the up-regulation of two types of nucleases (endonuclease S1/P1 and 3'-5' exonuclease), which may catalyze the degradation of RNA into shorter fragments [123, 124] and play a relevant role in nutrient mobilization under Pi-starvation. It seems likely that sRNA production upon Pi-starvation is an effect of RNA degradation by different types of nucleases. Thus, degraded RNA may serve as a source of Pi necessary in emerging plant organs. We found also two genes encoding oxalate oxidases, the expression of which was significantly downregulated during Pi-starvation. This class of genes is responsible for the inactivation of oxalic acid, which mediates fungal-plant pathogenesis in barley [125].

Conclusion

To conclude, our studies provide comprehensive data sets, which may serve as a rich platform for the characterization of barley responses to Pi-starvation at an RNA level. Furthermore, our data may be used as a reference tool for parallel studies in other crop plants.

Materials And Methods

Plant material

Three biological replicates of barley root and shoot samples were analyzed. One replicate consisted of three plants growing in a single pot containing 1.5 kg of soil mixed with sand in a 7:2 ratio. Material was collected from the barley line Rolap (obtained from the Institute of Plant Genetics of the Polish Academy of Sciences, Poznań, Poland [126]) growing under low-Pi (8 mg P/kg soil) and Pi-sufficient conditions (after addition of 60 mg P/kg soil), as described before [48]. On the 23rd day after sowing plant shoots (Zadoks decimal code 22–23 [127]), they were cut off and fresh tissue weight was measured. Immediately afterwards, shoots and roots were collected and frozen in liquid nitrogen to be kept at -80 °C until use.

Pi concentration measurements

Measurements of inorganic phosphate level were performed according to the protocol we have described before [46]. The samples were measured in two technical and three biological replicates using an Infinite F200 Pro (TECAN, Switzerland).

RNA isolation

Four procedures of RNA isolation were used, depending on the following experiments: (i) small RNA expression level analysis (ddPCR using TaqMan™ MicroRNA assays, NGS of small RNAs, Northern hybridization); (ii) transcriptome analysis; (iii) degradome - PARE (Parallel Analysis of RNA Ends) [128] analysis for mRNA cleaved by miRNA; or (iv) validation of RNA-Seq data using ddPCR.

(i) Small RNA expression level analysis

RNA isolation was performed using a modified method allowing enrichment of small RNAs, according to the detailed protocol we published before [64].

(ii) RNA for RNA-Seq

RNA was extracted from a 100 mg sample using RNA extraction buffer [104] and a Direct-Zol RNA MiniPrep Kit (Zymo Research). According to the Lexogen's SENSE mRNA-Seq Library prep kit v2 user guide DNase treatment step was omitted to avoid RNA hydrolysis.

(iii) RNA for degradome analysis

Procedure of RNA isolation from barley root and shoot (growing in low-Pi conditions) used for degradome profiling was performed using a method described by German et al. using RNA extraction buffer [128], along with some modifications that we have described previously [64, 129].

(iv) mRNA-Seq data validation

To validate the transcript level of significantly changed genes, we used precise dd-PCR analysis. To isolate RNA for these analyses, we used a Direct-Zol RNA MiniPrep Kit (Zymo Research) with some modifications that we have described in detail previously [104]. The RNA material was treated using DNase I enzyme from the above kit (Zymo Research).

Preparation of NGS libraries

We prepared three different NGS libraries: (i) small RNA, (ii) transcriptome - mRNA, and (iii) degradome.

(i) Small RNA libraries

Small RNA libraries were prepared using a TruSeq Small RNA Library Prep Kit (Illumina). In brief, small RNAs of 15–30 nt in length were separated on denaturing 8M urea 15 % polyacrylamide (PA) gel and purified and ligated to 3' and 5' RNA adapters. Next, the RNA fragments were reverse transcribed to run PCR: PCR products were indexed by utilization of specific RNA PCR Index Primers and PCR profile, according to the Illumina protocol (RPI, Illumina). PCR products were separated on 7 % PA gel containing 1 % glycerol. After 10' staining by SYBR™ Gold Nucleic Acid Gel Stain (Invitrogen, Thermo Fisher Scientific)/0.5xTBE buffer, DNA fragments of 140–160 bp in length were cut and eluted using 400 µl elution buffer (50 mM Mg-acetate, 0.5 M ammonium acetate, 1 mM EDTA, 0.1 % SDS) after O/N incubation, 28 °C, 400 rpm. Then, chloroform/phenol pH=8.0 purification libraries were precipitated using

1.5 ml GlycoBlue™ coprecipitant (15 mg/mL) (Ambion, Thermo Fisher Scientific) and three volumes of 100 % ethanol. Purified libraries were quantified using a Qubit® dsDNA HS Assay kit (Invitrogen, Thermo Fisher Scientific) and Qubit 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific). The quality of the libraries was analyzed using a High Sensitivity D1000 ScreenTape Assay (Agilent Technologies) and a 2200 TapeStation (Agilent Technologies). A total of 12 libraries were pooled together in equal molar ratio and sequenced by Fasteris SA (Switzerland).

(ii) Degradome library construction

Degradome library construction was performed according PARE technique described by German et al. [128]. Ligation was performed using a Rapid DNA Ligation Kit (Roche), according to the manufacturer instructions. The ligation mixture was composed of Mmel-digested PCR product and 3' DNA Adapter, kept for 6 hours at RT and at 4 °C overnight, then purified using phenol/chloroform extraction. PCR reaction was performed in a 50 µl volume containing Mmel fragment-3'Adapter template, appropriate index-containing primer (0.5 mM final concentration), Mmel Universal Fwd primer (0.5 mM final concentration), 350 mM dNTPs, Q5 reaction buffer, and Q5® Hot Start High-Fidelity DNA Polymerase (New England Biolabs), using the following steps: 94 °C for 2 min; 94 °C for 30 sec, 60 °C for 30 sec, 72 °C for 30 sec—14 cycles; and 72 °C for 7 min. PCR products were separated on 8 % PA gel containing 1 % glycerol. Appropriate in length bands were cut and eluted O/N. Quantitative analysis of the purified libraries was performed using a Qubit® dsDNA HS Assay kit (Invitrogen, Thermo Fisher Scientific) and a Qubit 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific). Finally, the quality of the libraries was analyzed using a High Sensitivity D1000 ScreenTape Assay (Agilent Technologies) and a 2200 TapeStation (Agilent Technologies). Each library possessed an individual specific index. The four libraries were pooled together in equal molar ratio and sequenced by Fasteris SA (Switzerland). In present study two degradome libraries were analyzed.

(iii) mRNA libraries

Transcriptome libraries were carried out using a SENSE mRNA-Seq library prep kit v2 (Lexogen), according to the manufacturer's protocol and as previously described [48]. A 420 pg of Spike-In RNA Variants SIRV-set3 (Lexogen) was added to 1500 ng of total RNA. ERCC mix was used for Spike-in analysis.

Library sequencing

Sequencing of small RNAs was performed (i) internally, using a MiSeq paired-end kit to check the library quality using a MiSeq® Reagent Kit v3 (Illumina) at the Laboratory of High Throughput Technologies, Adam Mickiewicz University, Poznań, Poland. Received data showed proper library quality and low-Pi induced changes in small RNA levels. (ii) The main deep sequencing (12 small RNA libraries, degradome, mRNA) was performed externally by Fasteris SA (Switzerland).

Data analysis

Differences in small RNAs, RNAs levels, and preliminary degradome data analysis were performed using a CLC Genomics Workbench (Qiagen Aarhus A/S).

Small RNA data analysis

The trimming procedure was used with default settings for quality trimming (quality score limit 0.02), adapter trimming, and for removal of small RNAs longer than 25 nt and shorter than 18 nt. Reads were extracted, counted, and normalized per 1,000,000 reads. Then, we set up the Experiment analysis for samples derived from roots and shoots separately (two-group comparison, unpaired). Empirical analysis of (EDGE) was used to find significant fold changes in small RNA expression levels between samples derived from different treated barley. Moreover, we performed EDGE Bonferroni and EDGE FDR p -value correction calculation. First, all small RNAs were annotated to miRBase (release 22) without mismatches and with strand-specific alignment. Then, unannotated small RNAs (i.e., those not identified in miRbase) were sorted according the lowest p -value. All identified differentially expressed sRNAs were annotated with internal ID numbers: IDs from 1 to 138 represent miRNAs identified in roots (p -value < 0.05), IDs 139–1934 represent other small RNAs identified in roots (Bonferroni p -value correction < 0.05), IDs 1935–2096 represent miRNAs identified in shoots (p -value < 0.05) and IDs represent 2097–2295 other small RNAs identified in shoots (Bonferroni p -value correction < 0.05).

Degradome data analysis

Degradome construction was performed using two different approaches, which allowed for a more in-depth analysis (**Fig. 2**). In the first method, the raw sequencing reads were processed by Cutadapt program (<https://cutadapt.readthedocs.io/en/stable/>) to trim low-quality and adapter sequences. Only sequences of length 15 nt and above were selected for further analyses. The processed sequencing reads were aligned to the reference sequences using bowtie. The count of 5'-end marked cleavage sites was scored by Perl script and normalized to the depth of sequencing and total signal for each of the reference transcripts. The putative miRNA:target pairs were predicted by a custom program (targetSeek) which included the following steps: (i) calculation of perfect match MFE (minimum free energy); (ii) RNAplex-based (Vienna package) screening for sRNA:transcript pairs; (iii) filtering number of bulges and length of sequence overhangs by MFE (percent of the perfect MFE match); and (iv) calculation of prediction score using a penalty schema for loops, bulges, and G:U wobble pairing. In the second approach, we used the PAREsnip2 software [130] to generate t-plots in conjunction with five databases (**Fig. 2**). Potential miRNA targets are classified into one of five categories, where category 0 indicates the best miRNA-target match. The lower the alignment score, the better the alignment between the sRNA and the target site [130]. During PAREsnip2 analysis, we set the Fahlgren and Carrington targeting rules to permit a mismatch or G:U wobble at position 10 [131].

Identification of DEGs

Experiments were performed in three biological replicates of plants grown under low-Pi and control conditions. Paired-end sequencing reactions of the 150 nt reads were performed using an Illumina

System. Total read numbers from six samples were mapped to the barley reference genome from Ensembl Plants Genes 42 (*Hordeum vulgare* IBSC v2). The library's quality and sequencing accuracy were verified carefully (i) by adding Spike-in RNA Variant Control Mixes (Lexogen) (**Additional file 24**) and (ii) by quality trimming. RNA-Seq analysis was performed using following normalization method - TPM expression values. TPM (Transcripts Per Million) is computed with the following equation $TPM = \frac{RPKM}{\sum RPKM} \times 10^6$. RPKM (Reads Per Kilobase of exon model per Million mapped reads) is computed using following equation $RPKM = \frac{Reads}{kb \times Million} \times 10^6$. Differentially expressed genes (DEGs) were selected based on fold change calculations ($p < 0.05$) and the selective Bonferroni test. RNA-Seq reads were mapped to the full-length transcripts using the CLC Genomics Workbench (QIAGEN) software, as previously described [48]. Among potential differentially expressed transcripts only those which went through restricted Bonferroni p -value correction were considered as differentially expressed genes (DEGs), which are listed in **Additional file 8**.

GO analysis

Gene ontology (GO) analyses were performed using the PANTHER *in silico* tool [132]. The over-representation binomial tests classified DEGs within GO domains (cellular component, biological process, and molecular function) with Bonferroni-corrected p -value < 0.05 .

cis-regulatory motif localization within DEG promoters

To analyze the enrichment of Pi-related *cis*-regulatory motifs, we extracted 2000 bps upstream of transcription start site from each identified DEG. Such data were directly screened to look for any either P1BS- or P-responsive PHO element consensus sequences using multiple promoter analysis with the PlantPAN3.0 tool (Chow et al. 2019).

ddPCR

To determine the absolute copy number of genes encoding *IPS1*, SPX-MFS1, endonuclease S1/P1, 3'-5' exonuclease, oxalate oxidase, and oxalate oxidase 2, we performed ddPCR using either EvaGreen Supermix (Bio-Rad) or TaqMan Assay (Bio-Rad) for mature miR827, according to the protocols previously described [48]. To normalize the copy number of miR827, we ran ddPCR for the *ARF1* reference gene using the TaqMan Assay id: AIMSIL4 (Thermo Fisher Scientific). Absolute gene expression was shown as normalized copy number per 1000 copies of the barley *ARF1* reference gene. All specific primers and probes (mature miR827, U6 snRNA) used in this paper are listed in **Additional file 25**.

Northern blot of mature miR827

To determine the mature miR827 expression level, we performed northern blot hybridization using a specific probe for analysis. All steps of these experiments were done according to a detailed protocol as described previously [64]. 10 µg of each RNA sample was run alongside a radioactively labelled Decade Marker (Invitrogen, Thermo Scientific) on a 15% polyacrylamide gel with 8M urea. The miR827 and U6 probe sequences are available in **Additional file 25**. The Decade Marker (Ambion) was loaded to control

the length of the tested RNAs. To calculate band intensity, we used the ImageQuant TL 8.1 software (GE Healthcare Life Sciences).

RNA-Seq data

Presented RNA-Seq data were submitted to GEO (NCBI) with the following accession number GSE145427, sRNA-Seq - GSE145425, transcriptome mRNA-Seq - GSE145426, degradome RNA-Seq - GSE145423.

Abbreviations

sRNA: small RNA; siRNA: small interfering RNA; Pi: inorganic phosphate; NGS: Next-Generation Sequencing; RNA-Seq: RNA Sequencing; DEM: differentially expressed miRNA; DES: differentially expressed small RNA; DEG: differentially expressed gene; GO: gene ontology; miRBase: the microRNA database; BLAST: Basic Local Alignment Search Tool; ddPCR: droplet digital Polymerase Chain Reaction; P1BS: PHR1 binding sequence; TF: transcription factor; PARE: Parallel Analysis of RNA Ends; EDGE: Empirical analysis of Differential Gene Expression; FDR: false discovery rate; MFE: minimum free energy; TPM: transcripts per million; RPKM: reads per kilo base of exon model per million mapped reads

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analyzed during the current study have been submitted to GEO database (accession numbers: GSE145423, GSE145425, GSE145426, GSE145427).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceptualization, writing - original draft preparation, writing - original draft editing, designing and preparing figures, plant material generation, Pi concentration measurements, ddPCR analysis, NGS data analysis, promoters' analysis, funding acquisition: P.S.; conceptualization, supervision and writing - original draft preparation, writing - original draft editing, NGS libraries preparation, NGS data analysis, degradome profiling, ddPCR analysis, Northern-blot analysis, funding acquisition: A.P.; NGS data analysis, visualization, preparing figures: D.B.; NGS libraries preparation K.K.; NGS libraries preparation P.N., degradome profiling W.K.; original draft review Z.S.K.

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Accession Numbers

Sequence data from this article can be found in the Ensembl Plants database for the barley genome: *IPS1* (HORVU4Hr1G079600), *SPX-MFS1* (HORVU2Hr1G094690), *SPX-MFS2* (HORVU6Hr1G065710), *GAMyb* transcription factor (HORVU3Hr1G079490), *PCF6* transcription factor (HORVU2Hr1G060120), *PHO2* (HORVU1Hr1G085570), *ARF1* (HORVU3Hr1G079700), 3'-5' exonuclease (HORVU6Hr1G007360), *S1/P1* endonuclease (HORVU2Hr1G112830), oxalate oxidase (HORVU4Hr1G005440), oxalate oxidase 2 (HORVU4Hr1G005380).

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Tables

Due to technical limitations, full-text HTML conversion of the Tables could not be completed. However, they can be downloaded and accessed as a PDF in the Supplementary Files.

Table 1. Degradome profiles for best scoring (score range 0-2) differentially expressed miRNAs (DEMs) identified in this study. Green color = DEMs from shoot, brown color = DEMs from root.

Table 2. List of genes predicted in degradome analysis to be guided for cleavage by the most up- and down-regulated sRNAs identified in this study.

Table 3. List of genes predicted in degradome analysis to be guided for cleavage by putative regulatory sRNAs (identified as DES) with best scoring matches.

Additional Files

Additional file 1. Normalized copy numbers of barley *IPS1* gene transcript in low-Pi treated root material. DdPCR was performed to examine the absolute gene expression of the barley *IPS1* gene. Obtained copy numbers were normalized per 1000 copies of the *ARF1* reference gene transcript. Asterisks indicate a significant differences (**p*-value < 0.05) calculated using two-tailed Student's *t*-tests.

Additional file 2. Characteristic of reads obtained from small RNA deep sequencing.

Additional file 3. MicroRNAs and small RNAs (other) for which expression is significantly changed during Pi-starvation in barley roots and shoots. ID numbers 1–138: miRNAs identified in roots (p -value < 0.05); ID numbers 139–1934: other small RNAs identified in roots (Bonferroni p -value correction < 0.05); ID numbers 1935–2096: miRNAs identified in shoots (p -value < 0.05); ID numbers 2097–2295: other small RNAs identified in shoots (Bonferroni p -value correction < 0.05). Samples R4–R6 = low-Pi root; R16–R18 = control; S4–S6 = low-Pi shoot; S16–S18 = control shoot. NaN means "Not a Number", describing molecules that were exclusively expressed in low-Pi or control samples. Data created using CLC Genomics Workbench.

Additional file 4. List of differentially expressed other small RNAs (ID 2097–2295) in barley shoots (low-Pi vs. control) identified in this study (Bonferroni corrected p -value < 0.05). Based on the available Ensembl Plants database, we classified each sequence into best-matching functional classes of cDNAs.

Additional file 5. List of differentially expressed other small RNAs (ID 139–1934) in barley roots (low-Pi vs. control) identified in this study (Bonferroni corrected p -value < 0.05). Based on the available Ensembl Plants database, we classified each sequence into best-matching functional classes of cDNAs.

Additional file 6. Length distribution of DESs identified in barley roots and shoots.

Additional file 7. Annotation distribution of DESs identified in barley roots and shoots.

Additional file 8. List of 98 DEGs from barley shoots (low-Pi vs. control/sufficient Pi) identified in this study.

Additional file 9. Upstream sequences (2 kb) extracted from all 98 DEGs used for *cis*-regulatory motif prediction analysis.

Additional file 10. List of identified P1BS motifs within the DEG promoters.

Additional file 11. List of identified P-responsive PHO motifs within the DEG promoters.

Additional file 12. Degradome profile (TargetSeek approach) demonstrates potential mRNA targets for differentially expressed miRNAs (DEMs) identified in barley shoots (low-Pi vs. control). The lower the alignment score, the more reliable the prediction.

Additional file 13. Degradome profile (TargetSeek approach) demonstrates potential mRNA targets for differentially expressed other sRNAs (DESs) identified in barley shoots (low-Pi vs. control). The lower the alignment score, the more reliable the prediction.

Additional file 14. Degradome profile (TargetSeek approach) demonstrates potential mRNA targets for differentially expressed miRNAs (DEMs) identified in barley roots (low-Pi vs. control). The lower the alignment score, the more reliable the prediction.

Additional file 15. Degradome profile (TargetSeek approach) demonstrates potential mRNA targets for differentially expressed other sRNAs (DESS) identified in barley roots (low-Pi vs. control). The lower the alignment score, the more reliable the prediction.

Additional file 16. Degradome profile (PAREsnip2 approach) demonstrates potential mRNA targets for differentially expressed miRNAs (DEMs) identified in barley shoots (low-Pi vs. control). The lower the alignment score, the more reliable the prediction.

Additional file 17. The t-plots generated by PAREsnip2 software showing the potential mRNA targets for differentially expressed miRNAs (DEMs) identified in barley shoots (low-Pi vs. control).

Additional file 18. Degradome profile (PAREsnip2 approach) demonstrates potential mRNA targets for differentially expressed other sRNAs (DESS) identified in barley shoots (low-Pi vs. control). The lower the alignment score, the more reliable the prediction.

Additional file 19. The t-plots generated by PAREsnip2 software showing the potential mRNA targets for differentially expressed other sRNAs (DESS) identified in barley shoots (low-Pi vs. control).

Additional file 20. Degradome profile (PAREsnip2 approach) demonstrates potential mRNA targets for differentially expressed miRNAs (DEMs) identified in barley roots (low-Pi vs. control). The lower the alignment score, the more reliable the prediction.

Additional file 21. The t-plots generated by PAREsnip2 software showing the potential mRNA targets for differentially expressed miRNAs (DEMs) identified in barley roots (low-Pi vs. control).

Additional file 22. Degradome profile (PAREsnip2 approach) demonstrates potential mRNA targets for differentially expressed other sRNAs (DESS) identified in barley roots (low-Pi vs. control). The lower the alignment score, the more reliable the prediction.

Additional file 23. The t-plots generated by PAREsnip2 software showing the potential mRNA targets for differentially expressed other sRNAs (DESS) identified in barley roots (low-Pi vs. control).

Additional file 24. Spike-in quality control of RNA-Seq samples from barley shoots (low-Pi vs. control). Correlation between known and measured spike-in concentrations.

Additional file 25. List of primers and probes used in this study.

Additional file 26. Original, full-length blot of mature hvu-miR827 analysis. Lane 1: Decade™ Marker System (Invitrogen, Thermo Fisher Scientific); Lane 2: empty space (no sample loaded); Lane 3-5: RNA samples from root (Pi sufficient); Lane 6-8: shoot (Pi sufficient); Lane 9-11: root (low-Pi); Lane: 12-14: shoot (low-Pi).

Additional file 27. Original, full-length blot of U6 snRNA analysis. Lane 1: Decade™ Marker System (Invitrogen, Thermo Fisher Scientific); Lane 2: empty space (no sample loaded); Lane 3-5: RNA samples

from root (Pi sufficient); Lane 6-8: shoot (Pi sufficient); Lane 9-11: root (low-Pi); Lane: 12-14: shoot (low-Pi).

Figures

Figure 1

The validation of barley line Rolap plant material under low-Pi stress. (A) Pictures of the plants (n = 3) collected on the 23rd day after sowing, grown under low-Pi, 8 mg P/kg soil (left) and control-Pi, addition of 60 mg P/kg soil (right), conditions. (B) Shoot fresh tissue weight (n = 3). (C) The Pi concentration measurements performed for barley roots and shoots (n = 3). Asterisks indicate a significant difference (*p-value < 0.05) calculated using two-tailed Student's t-tests. Scale bar = 10 cm. Error bars = SD.

^a Ensembl Plants v.40; *Hordeum vulgare* genes (IBSC v2), 42 859 genes classified as: (i) antisense RNA – 22, (ii) nontranslating RNA – 2 340, (iii) pre-miRNA – 77, (iv) protein coding – 37 705, (v) pseudogene – 100, (vi) RNase – 1, (vii) rRNA – 1 385, (viii) sense intronic – 173, (ix) snoRNA – 455, (x) snRNA – 192, (xi) SRP_RNA – 11, (xii) tRNA - 688

^b (i) microRNA reads with significant p-value from roots and shoots, (ii) small RNA reads with significant (Bonferroni corrected) p-value from roots and shoots, (iii) protein coding cDNAs annotated to Ensembl Plants database, (iv) transcripts of 98 genes with significant expression changed upon low-Pi and (v) degradome profiles from roots and shoots

Figure 2

The framework illustrating the data generation protocols used in this study. The low-Pi stress-specific subsets of RNAs were generated following (i) deep sequencing of small RNAs from barley shoots and roots, (ii) transcriptomic RNA-Seq for barley shoots, and (iii) degradome profiling for barley shoots and roots. The obtained data sets were mapped to the references collected from miRBase and Ensembl Plants database exclusively for barley. The log2 scale for fold change and Bonferroni corrections were calculated to pick the significantly changed sequences under Pi-deficient and Pi-sufficient conditions.

Figure 3

Differentially expressed microRNAs (DEMs) in barley plants under low-Pi regime. (left panel, A) Venn's diagram illustrating the number of identified DEMs with p -value < 0.05 (left panel). The reduced list of DEMs that passed the Bonferroni test is presented in the right panel. The ID specifies the miRNA sequence, according to data sets obtained in NGS (Additional file 3). The given fold change is shown as a $\log_2$ value in the column $\log_2(\text{fc})$. Targeta = TargetSeek approach; Targetb = PAREsnip2 approach.

Predicted target genes are presented in the table based on our dual degradome profiling (Additional files 13, 15, 17, 21). (right panel, A) Type categorizes miRNAs based on the sequences deposited in miRBase without mismatches, isomiRs include miRNAs with nucleotide shift (super or sub) at their 5' , 3', or at both ends [133]. Yellow dots mark miRNAs differentially expressed in both organs; shoot ID 2019 corresponds to root ID 75 and, similarly, ID 2072 = ID 113, ID 2063 = ID 105, and ID 2073 = ID 114. (B) The absolute gene expression quantification of identified mature hvu-miR827 and its predicted target gene SPX-MFS1 using ddPCR. The bars represent copy numbers normalized to 1000 copies of the ARF1 reference gene; *p-value < 0.05, calculated using two-tailed Student's t-tests for three biological and two technical replicates. Error bars = SD. (C) Detection of hvu-miR827 expression pattern in barley samples used in this study for NGS analysis. Specific probes for hvu-miR827 mature sequence and U6 reference gene were used for Northern hybridization performed on a single membrane. The number represents hvu-miR827 band intensity compared to U6 snRNA. The blots were cropped and original, full-length blots are presented in Additional file 26 and Additional file 27. (D) Degradome profile of SPX-MFS1 gene generated with DEMs from barley shoot using the PAREsnip2 approach. The red vertical line shows the cleavage position directed by hvu-miR827; the cleavage position 192 is within exon no. 2 in the 5'-UTR of the SPX-MFS1 transcript (p-value = 0.014). The black vertical lines show the positions within the SPX-MFS1 cDNA to which degradome fragments (reads) were mapped. The number of reads (fragment abundance) is depicted by the height of the red and black lines. Below the graph, the structure of the SPX-MFS1 transcript is presented. The white boxes denote UTRs, the gray boxes denote coding sequence, and the red dotted line denote the cleavage site within the 5'-UTR.

Figure 4

Differentially expressed other small RNAs (DESRs) in barley plants under the low-Pi regime. (A) Venn's diagram illustrating the quantity of identified DESRs with Bonferroni corrected p -value (left panel). The annotation distribution of DESRs in barley shoots and roots based on the calculations present in Additional file 7 (right panel). (B) The length distribution of DESRs in roots and shoots. (C) Degradome profiles identified two DESRs that are significantly changed in shoot or root and exhibit high possibility to cleave mRNA targets. The HORVU7Hr1G053570 locus encodes an uncharacterized protein with unknown PTHR47188 domain; the HORVU7Hr1G003920 locus encodes glutaminyl-peptide cyclotransferase. The white boxes denote UTRs, the gray boxes denote coding sequence, and the red dotted line denote cleavage site.

Figure 5

Functional and genomic analysis of 98 differentially expressed genes in barley shoots under low-Pi. (A) The significantly enriched GO terms in the categories of biological process, molecular function, and cellular localization. The Bonferroni adjustment was applied to correct the p-values. (B) Chromosomal mapping of 98 DEGs identified in this study. Lower panel illustrates the percentage between quantitative distribution of either up-regulated or down-regulated genes under low-Pi conditions and total number of

protein-coding genes in each barley chromosome. Scale bar for chromosomes = 160 Mbp. (C) Summarized list of ten most up-regulated and ten most down-regulated genes found in RNA-Seq analysis. Full list containing all 98 genes with their accession numbers is available in Additional file 8.

Figure 6

Molecular characterization of identified DEGs from barley shoot. (A) Quantification by ddPCR of the absolute expression levels of the DEGs belonging to two selected pathways in barley shoots. Two up-

regulated DEGs, which encode endonuclease S1/P1 and 3'-5' exonuclease, are involved in nucleic acid metabolism and further RNA degradation. Two down-regulated DEGs, which encode two different oxalate oxidases, are involved in the reduction of oxidative stress. The bars represent copy numbers normalized to 1000 copies of the ARF1 reference gene; *p-value < 0.05, **p-value < 0.001, calculated using two-tailed Student's t-tests for three biological and two technical replicates. (B) Localization of all P1BS and P-responsive PHO cis-regulatory elements within the 2000 bp upstream from the DEG TSSs. On the graph, grouped motifs are specifically located in every 500 bp, induced ($\log_2(\text{Fch}) > 0$), or repressed ($\log_2(\text{Fch}) < 0$). The motif quantity in each group is shown in either red (P1BS) or blue (PHO elements) dots.

Figure 7

Barley pathways triggered by Pi-starvation to maintain plant homeostasis. Graphical overview illustrates primary strategies in Pi-starved barley plants based on our shoot transcriptomic analysis, small RNA-Seq, and degradome profiling. The low-Pi induced feedback loop is located in the middle part, which is involved in the positive regulation of phosphate transporters (i.e., PHT1, PHO1) prompting Pi uptake. The MYB-coiled-coil (MYB-CC) protein family includes PHR transcription factors (yellow frame), which act as a major regulator to either induce or repress Pi-responsive genes in plants. The asterisk represents the

data from shoot and root sRNA-Seq. Dotted lines display wide area of molecular networks, connecting most of the plant low-Pi stress responses. All components depicted on the graph are listed in Additional file 8. Values correspond to $\log_2(\text{fold change})$ with Bonferroni corrected p-values.

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