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Histone H2A.Z Regulates the Expression of Several Classes of Phosphate Starvation Response Genes But Not as a Transcriptional Activator\textsuperscript{1}[OA]

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Phosphate (Pi) availability is a major constraint to plant growth. Consequently, plants have evolved complex adaptations to tolerate low Pi conditions. Numerous genes implicated in these adaptations have been identified, but their chromatin-level regulation has not been investigated. The nuclear actin-related protein ARP6 is conserved among all eukaryotes and is an essential component of the SWR1 chromatin remodeling complex, which regulates transcription via deposition of the H2A.Z histone variant into chromatin. Here, we demonstrate that ARP6 is required for proper H2A.Z deposition at a number of Pi starvation response (PSR) genes in Arabidopsis (Arabidopsis thaliana). The loss of H2A.Z at these target loci results in their derepression in arp6 mutants and correlates with the presence of multiple Pi-starvation-related phenotypes, including shortened primary roots and increases in the number and length of root hairs, as well as increased starch accumulation and phosphatase activity in shoots. Our data suggest a model for chromatin-level control of Pi starvation responses in which ARP6-dependent H2A.Z deposition modulates the transcription of a suite of PSR genes.

All living things require phosphorus (P) for survival. Consequently, organisms possess regulatory programs that control the onset of events aimed at facilitating P conservation and/or acquisition during P limitation. The Saccharomyces cerevisiae PHO regulon includes a two-component system consisting of regulatory proteins and transcriptional activators that mediates coordinated responses to P limitation (Oshima, 1997). Perception of low Pi conditions promotes the binding of the basic helix-loop-helix transcription factor, Pho4p, to PHO box cis-elements in the promoters of many structural genes, including phosphate transporters and phosphatases (Oshima, 1997; Persson et al., 2003; Nishizawa et al., 2008).

1 This work was supported by grants to R.B.M. from the National Institutes of Health (GM 36397–22) and the U.S. Department of Energy’s Office of Biological and Environmental Research (DEFG0203ER63620) to K.G.R. from the U.S. Department of Energy’s Office of Biological and Environmental Research (DEG0796ER20257) and Environmental Remediation Sciences Division (DEFC2002ER66020) to K.G.R. from the U.S. Department of Energy.

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www.plantphysiol.org/cgi/doi/10.1104/pp.109.145532

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structure at promoters and other DNA regulatory elements to modulate both basal and induced transcription rates. A number of studies have shown that several chromatin-level mechanisms are involved in regulating the transcription of some yeast structural PHO genes. For example, the SWI/SNF nucleosome remodeling complex and the Gcn5 histone acetyltransferase are necessary for full induction of several phosphatase genes (PHO5, PHO8, PHO11, and PHO12) and the PHO84 high-affinity phosphate transporter gene (Santisteban et al., 2000; Sudarsanam et al., 2000; Barbaric et al., 2007; Wippo et al., 2009). The SWR1 complex, which regulates transcription through the deposition of the H2A.Z histone variant at target genes, has also been implicated in modulating the transcription of a number of PHO genes (Santisteban et al., 2000; Lindstrom et al., 2006).

In contrast to yeast, the involvement of chromatin-level mechanisms in the regulation of plant PSR genes has not been investigated. Recently, several studies have indicated that the SWR1 complex described in other eukaryotes is conserved in Arabidopsis (March-Diaz and Reyes, 2009). In addition, the Arabidopsis nuclear actin-related protein ARP6 has been identified as a key component of SWR1 and is required for normal deposition of histone H2A.Z at target loci (Choi et al., 2007; Deal et al., 2007). As a result, plants defective in ARP6 exhibit diverse developmental phenotypes. For example, mutation of ARP6 results in altered leaf, inflorescence, and flower development and early flowering (Choi et al., 2005; Deal et al., 2005). The early flowering phenotype is due, in part, to disruption of ARP6-dependent H2A.Z deposition at the floral repressor genes FLOWERING LOCUS C, MADS AFFECTING FLOWERING4 (MAF4), and MAF5 (Deal et al., 2007). Previously, we observed that arp6 mutant seedlings also exhibit altered root growth similar to that of wild-type Arabidopsis grown under Pi-replete conditions (March-Diaz and Reyes, 2009). Our data support a role for ARP6 in modulating PSRs by regulating expression of key PSR genes.

The goal of this study was to investigate the role of ARP6-dependent H2A.Z deposition in controlling PSRs in Arabidopsis. We report that mutation of ARP6 results in a dramatic decrease in H2A.Z abundance at a number of PSR genes from diverse functional categories. The loss of H2A.Z is accompanied by increases in gene transcription (i.e. derepression) of the PSR genes and the manifestation of multiple PSRs in arp6 mutant seedlings. Our data support a role for the SWR1 complex in repressing the onset of PSRs by modulating the expression of a number of PSR genes.

RESULTS

arp6 Mutants Exhibit PSRs under Pi-Replete Conditions

Plants have evolved complex adaptations to tolerate low Pi conditions. Some of the most prominent PSRs are changes in root morphology aimed at mining additional Pi from soil (Franco-Zorrilla et al., 2004). These include increases in the number and length of root hairs and a decrease in primary root growth (Ma et al., 2001; Sanchez-Calderon et al., 2005; Jain et al., 2007). In preliminary experiments, we observed that arp6 mutant seedlings exhibited altered root growth similar to that of Pi-starved wild type. To initiate an investigation of the potential role of ARP6 in modulating PSRs, seedlings of the arp6 T-DNA mutants arp6-1 and arp6-2 (Deal et al., 2005) were grown under Pi-replete conditions and were screened for traits indicative of Pi starvation at 7 or 12 d old. Seedlings of both arp6-1 and arp6-2 had approximately 2.5-fold more root hairs relative to the wild type in a 5-mm section from the primary root apex, and these hairs were 2-fold longer on average (Fig. 1, A and B). In addition, both arp6 mutants exhibited reduced primary root growth relative to the wild type (Fig. 1C). Other PSRs include increased starch accumulation, which results from low Pi-induced alteration of primary metabolism (Amtmann et al., 2006), and increased acid phosphatase activity, which aims to remobilize intracellular Pi (del Pozo et al., 1999; Li et al., 2002). In shoots of the arp6 mutants, we observed increases in both starch accumulation (Fig. 1D) and acid phosphatase activity (Fig. 1E) compared to the wild type. Together, these data suggest the onset of PSRs in arp6 mutant seedlings despite being grown under Pi-replete conditions. To determine if the PSRs in the mutants correlate with a defect in cellular Pi status, we compared the soluble Pi contents of arp6-1, arp6-2, and the wild type. In both shoots and roots, arp6-1 and arp6-2 seedlings contained moderately less Pi compared to the wild type (Fig. 2).

Molecular Phenotypes of arp6 Mutant Seedlings

To gain insight into the factors influencing the Pi-starvation phenotypes we observed in arp6 mutants, we used quantitative reverse transcription-PCR to compare the transcript levels from a number of PSR genes (Misson et al., 2005; Bari et al., 2006) among 12-d-old wild-type, arp6-1, and arp6-2 seedlings. Specifically, we examined six different functional categories of Pi-starvation-induced genes, including those involved in Pi transport, Pi distribution, signal transduction, primary metabolism (e.g. lipid and starch metabolism), and protein turnover (Table I). First, we quantified the Pi starvation induction of these genes in both shoots and roots of wild-type seedlings. Transcript levels for all 10 genes were significantly higher (i.e. derepressed) in both tissues of Pi-starved wild type (i.e. 5 d on normal media followed by 7 d on Pi-deficient media; see “Materials and Methods”) compared to the wild type grown under Pi-replete conditions (Table I), corroborating data from previous studies (Misson et al., 2005; Bari et al., 2006). Next, we quantified the expression of these genes in the wild type, arp6-1, and arp6-2 grown under Pi-replete conditions. Transcript levels for all but two of the 10 genes were at least 2-fold greater in

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and arp6-2 than in the wild type in at least one tissue (i.e., shoot or root; Fig. 3). For instance, expression of At4, which is involved in Pi distribution (Shin et al., 2006), was substantially greater (from 21- to 83-fold) in shoots and roots of both arp6 mutants compared to the wild type. In addition, the shoot expression of β-amylase (BMY1), which plays a role in starch metabolism (Mita et al., 1997; Niewiadomski et al., 2005) was approximately 5-fold higher in both mutants relative to the wild type. This correlates with the increased starch accumulation observed in arp6-1 and arp6-2 shoots grown under Pi-replete conditions (Fig. 1D). Taken together, these results suggest that ARP6 plays a significant role in regulating PSRs in Arabidopsis.

Pi signaling pathways in plants are largely unknown, although some regulatory factors have been identified. A subcomponent of the Arabidopsis Pi signaling network involves the key MYB factor PHR1 as a transcriptional activator of a microRNA, mir399, which regulates the E2 conjugase, PHO2. PHO2, in turn, controls expression of a subset of PSR genes, including At4, IPS1, Pht1;8, and Pht1;9 (Bari et al., 2006). One explanation for the derepression of multiple PSR genes in arp6 mutants could be a defect in the PHR1-mir399-PHO2 signaling pathway. To test this, we compared the expression of PHR1, mir399 (via analysis of one of the five mir399 primary transcripts, mir399c; Bari et al., 2006), and PHO2 among the wild type, arp6-1, and arp6-2. As shown in Figure 4, we saw no significant changes (<2-fold) in transcript levels for these genes in either shoots or roots between the wild type and the arp6 mutants. This suggests that the PHR1-mir399-PHO2 signaling pathway does not play a major role in the derepression of PSR genes in arp6-1 and arp6-2 under Pi-replete conditions.

Figure 1. arp6 mutants exhibit Pi starvation symptoms under Pi-replete conditions. Data for 7- (A and B) and 12-d-old (C–E) wild-type (WT), arp6-1, and arp6-2 seedlings are shown. A and B, Development of root hairs in a 5-mm section from the primary root tip. Bar = 1 mm. C, Primary root length. D and C, Values are the mean ± se; n = 20 (10 seedlings from each of two independent experiments). D, Representative iodine-stained shoots of 10 seedlings each of the wild type and the arp6 mutants showing starch accumulation. E, Phosphatase activity in the shoots of the wild type and the arp6 mutants. Values are the mean ± se; n = 9 replicates of 150 to 200 seedlings each. Bars with asterisks are significantly different from the wild type (t test, P ≤ 0.05).

PSR Genes Are Targets of H2A.Z Histone Variant Deposition

Arabidopsis ARP6 participates in a SWR1-like chromatin remodeling complex that deposits the H2A.Z histone variant into chromatin (Deal et al., 2007;...
March-Diaz et al., 2007). To determine whether PSR genes are directly targeted by ARP6-dependent H2A.Z deposition, we performed chromatin immunoprecipitation (ChIP) analyses on 10 PSR genes in 12-d-old wild-type and arp6-1 seedlings using Arabidopsis H2A.Z antibodies. ChIP with H2A.Z antibodies significantly enriched all 10 loci in both shoots and roots from the wild type compared to control antisera but did not enrich these loci from arp6-1 tissues (Fig. 5). These results indicate that H2A.Z is deposited at all the PSR genes tested under normal conditions and that ARP6 is required for this deposition.

If H2A.Z is involved in the regulation of PSR genes, its presence at target loci may be dependent on the Pi status of the plant. To test this, we used ChIP analysis to compare the abundance of H2A.Z at the 10 PSR genes between Pi-replete and Pi-starved wild-type seedlings. Again, H2A.Z antibodies significantly enriched all 10 loci in Pi-replete wild type; however, the H2A.Z enrichment for five of the genes was significantly lower in Pi-starved wild type (Fig. 6). This indicates that the levels of H2A.Z at a subset of the PSR genes examined were lower during transcriptional activation than under repressive conditions.

**DISCUSSION**

As in other eukaryotes, the SWR1 chromatin remodeling complex is required for deposition of histone H2A.Z at target loci in Arabidopsis (Choi et al., 2007; Deal et al., 2007; March-Diaz and Reyes, 2009), and examination of H2A.Z localization genome-wide suggests that this histone variant is involved in the modulation of numerous genes (Zilberman et al., 2008). The nuclear actin-related protein ARP6 has been identified as a key component of SWR1, and as a result, mutation of ARP6 results in a pleiotropic phenotype, causing a variety of defects, including altered development of leaf, inflorescence, and flower, as well as early flowering (Choi et al., 2005; Deal et al., 2005). In contrast, ARP6 overexpression lines are phenotypically identical to the wild type with respect to flowering time, leaf, root, inflorescence, and flower development, and fertility (Deal et al., 2005; R.B. Deal, A.P. Smith, and R.B. Meagher, unpublished data). This is likely due to the fact that ARP6 activity is limited by other components of the SWR1 complex. A similar phenomenon has been observed for another SWR1-complex factor, SERRATED AND EARLY FLOWERING/AtSWC6. Loss of AtSWC6 causes many of the same developmental defects as mutation of ARP6 (Choi et al., 2007; March-Diaz et al., 2007), yet AtSWC6 overexpression also confers no visible or molecular phenotypes (Choi et al., 2007).

The early flowering phenotype in arp6 mutants is due, in part, to disruption of ARP6-dependent H2A.Z deposition at several floral repressor genes (Deal et al., 2007). Preliminary microarray experiments comparing wild-type and arp6-1 plants reveal large numbers of genes that are either up-regulated, down-regulated, or unchanged (data not shown). However, further examination of a subset of genes whose expression was reduced in arp6-1, including a CONSTANS-LIKE transcription factor, the WKR70 transcription factor, and the putative disease resistance genes PRB1 and TIR, indicated that even in wild-type plants these loci did not accumulate significant amounts of H2A.Z (Deal et al., 2007), suggesting that these and perhaps many other genes are not directly regulated by ARP6-dependent H2A.Z deposition. Here, we demonstrate that mutation of ARP6 results in many morphophysiological and molecular phenotypes indicative of PSRs. Together, these phenotypes, along with our expression and ChIP analyses, suggest that ARP6 directly modulates the manifestation of PSRs through its role in the SWR1 complex, which deposits H2A.Z at a number of PSR genes.

Data from a wealth of studies support a positive role for H2A.Z in transcription by poising genes for acti-

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**Table 1. Pi starvation induction of PSR genes in wild-type seedlings**

Relative quantity (RQ) values are the mean ± se from three technical replicates where the fold inductions were calculated by normalizing expression from Pi-starved tissues (P−) to expression from the wild type grown under Pi-replete conditions (P+).

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In contrast, here, we show that loss of H2A.Z at many PSR genes coincides with their derepression, suggesting a negative role for H2A.Z in gene regulation. This phenomenon may result from an overriding transcriptional activation mechanism(s) initiated by Pi starvation of arp6 mutant tissues that does not require H2A.Z. However, the Pi levels in arp6 tissues were 83 to 91% of wild-type levels (Fig. 2), consistent with the arp6 mutants experiencing only a small Pi starvation stress. Alternatively, H2A.Z may play a repressive role in the transcription of some PSR genes. Gene expression studies in H2A.Z-deficient mutants of S. cerevisiae (htz1Δ; Meneghini et al., 2003) and Arabidopsis (hta9/hta11; March-Diaz et al., 2008) identified substantial numbers of genes that were either up-regulated or down-regulated, suggesting that H2A.Z plays both repressive and inductive roles in transcription. In addition, our ChIP data show that for a subset of PSR genes examined, the levels of H2A.Z were higher at loci in Pi-replete tissues compared to Pi-starved tissues (Fig. 6). Other studies have also shown that H2A.Z is more abundant at some target loci under repressive conditions than during activation (Guillemette et al., 2005; Raisner et al., 2005; Deal et al., 2007). Furthermore, H2A.Z has been implicated in the repression of a number of stress response genes in yeast (Lindstrom et al., 2006) and in the repression of the systemic acquired resistance response in Arabidopsis (March-Diaz et al., 2008). Therefore, under normal conditions (i.e. Pi replete), ARP6-mediated H2A.Z deposition may act to maintain some PSR genes in a repressed transcriptional state.

Although H2A.Z appears to have a negative impact on PSR gene expression, the associated mechanisms are unclear. Under normal conditions, H2A.Z-containing nucleosomes might create a local chromatin structure at target loci that is not conducive to gene expression, perhaps by interfering with the binding of transcriptional activators and/or by promoting transcriptional repressor binding. A number of both positive and negative PSR-related transcription factors are known (Rubio et al., 2001; Yi et al., 2005; Chen et al., 2007; Devaiah et al., 2007a, 2007b; Duan et al., 2008), and their activities could be dependent on local chromatin environments influenced by H2A.Z. Our molecular analyses demonstrated that transcript levels for PHR1, miR399, and PHO2 were unchanged in arp6 mutants. However, PHR1 expression is not highly responsive to Pi starvation even though it plays a key role in activating downstream PSR genes (Rubio et al., 2001). Therefore, our analyses do not rule out the possibility that loss of H2A.Z in arp6 mutants impacts the interaction of the PHR1 protein with the promoters of H2A.Z-targeted PSR genes. Nucleosome remodeling at the promoters of several yeast PHO

Figure 3. Multiple PSR genes are misregulated in arp6 mutants. The tissues (shoots and roots) examined were harvested from 12-d-old seedlings of the wild type (WT), arp6-1, and arp6-2 grown under Pi-replete conditions. Values are plotted on a log scale and are the mean ± se from two biological replicates (of three technical replicates each) where the fold changes were normalized to transcript levels in the wild type. ND, no data; Pht1;2 transcripts in shoot tissues were below the limit of detection in these experiments.

Figure 4. Expression of PHR1-miR399-PHO2 signaling pathway components in arp6 mutants. The tissues (shoots and roots) examined were harvested from 12-d-old seedlings of the wild type (WT), arp6-1, and arp6-2 grown under Pi-replete conditions. Values are the mean ± se from two biological replicates (of three technical replicates each) where the fold changes were normalized to transcript levels in the wild type.
genes (e.g. PHO5, PHO8, and PHO84) occurs in response to Pi starvation and exposes cis-elements recognized by the Pho4p transcriptional activator (Wippo et al., 2009). A similar mechanism that involves H2A.Z may be in place for the regulation of Arabidopsis PSR genes. However, a direct comparison cannot be made since the Arabidopsis PSR genes examined in this study are derepressed in H2A.Z-defective plants (i.e. arp6 mutants), whereas loss of H2A.Z results in the down-regulation of the yeast PHO genes (Santisteban et al., 2000; Lindstrom et al., 2006). The expression of yeast PHO5, PHO8, and PHO84 all have requirements for the SWI/SNF remodeling complex, the Gcn5 histone acetyltransferase, and the Asf1 histone chaperone, but to considerably different degrees (Barbaric et al., 2007; Wippo et al., 2009). Microarray analyses have also implicated SWR1, the NuA4 histone acetyltransferase complex, and the Isw1 chromatin-remodeling enzyme in the regulation of these and other PHO genes (Lindstrom et al., 2006). In addition, regulation of gene transcription by H2A.Z is influenced by the ability of H2A.Z to antagonize DNA methylation-induced gene silencing (Zilberman et al., 2008). The fact that H2A.Z is one of many components that regulates gene transcription likely explains the observation that although H2A.Z is found genome-wide, its loss affects the transcription of only a subset of the genome (Lindstrom et al., 2006; Zilberman et al., 2008). This notion is supported by our data showing that the expression of some H2A.Z-targeted PSR genes (e.g. Phl1;1 and Phl1;4) is unchanged in arp6 mutants (compare Figs. 3 and 5). Differences in the relative PSR gene transcript levels in arp6 tissues (Fig. 3) compared to their levels in Pi-starved wild type (Table I) further suggest varying dependence of PSR gene promoters on H2A.Z. This may also reflect differences in the extent to which H2A.Z modulates basal and induced expression at distinct genes. Our ChIP data comparing

**Figure 5.** ARP6 is required for H2A.Z deposition at a suite of PSR genes. The abundance of histone variant H2A.Z was assayed in 12-d-old seedlings (shoots and roots) of the wild type (WT) and arp6-1 for 10 PSR genes using a ChIP assay with H2A.Z antibodies. The values are the average fold enrichment of DNA ± s.d as measured by real-time PCR of six replicates from two independent experiments.

**Figure 6.** The presence of histone variant H2A.Z at many PSR genes differs between Pi-replete and Pi-starved wild type. The abundance of histone variant H2A.Z was assayed at 10 PSR genes using a ChIP assay with H2A.Z antibodies. The tissues examined were harvested from wild-type seedlings grown hydroponically for 12 d in complete nutrient media and transferred either to fresh nutrient media (P+) or to nutrient media lacking Pi (P−) for an additional 2 d. The values are the average fold enrichment of DNA ± s.d as measured by real-time PCR of six replicates from two independent experiments.
Pi-replete and Pi-starved wild type, which show a difference in H2A.Z abundance at only some of the PSR genes tested, provide additional evidence for promoter specificity in regard to regulation by H2A.Z (Fig. 6). Alternatively, the Pi starvation treatment employed in our study may not yield maximal induction for all PSR genes. Future investigation of PSR gene transcription in arp6 mutants grown under different Pi concentrations, as well as examination of genetic interactions between SWR1 components and other chromatin cofactors, will help to elucidate the relative importance of H2A.Z in controlling basal and Pi-starvation-induced PSR gene transcription.

Our data indicate that ARP6 is required for proper H2A.Z deposition at a number of diverse PSR genes, suggesting that H2A.Z plays a significant role in modulating responses to Pi starvation in Arabidopsis. This phenomenon shares similarities with the PHO regulatory system in yeast, where transcription of many structural genes is regulated by H2A.Z. An important distinction is that loss of H2A.Z results in the down-regulation of yeast PHO genes but a derepression of the Arabidopsis PSR genes. However, a microarray study in yeast revealed that at least one PHO gene encoding a phosphate transporter (Pho87) is derepressed in the H2A.Z (htz1) mutant (Lindstrom et al., 2006). In addition, loss of Set1, the catalytic subunit of a histone methylation complex, results in the derepression of PHO5 and PHO84 (Carvin and Klade, 2004). These studies suggest that gene repression by chromatin cofactors plays a role in modulating PSRs in Arabidopsis, as well as in yeast. It is possible that both yeast and plants utilize chromatin-level mechanisms, such as SWR1, to repress multiple stress-induced pathways under normal conditions. Mutation of yeast SWR1 and/or H2A.Z (HTZ1) results in the derepression of a large number of stress-induced genes, including many heat shock genes (Lindstrom et al., 2006). In addition, mutation of PIE1, the Arabidopsis SWR1 homolog, results in the derepression of numerous stress-responsive genes (March-Diaz et al., 2008). A number of these genes are defense related, implicating the SWR1 complex in the repression of the systemic acquired resistance response (March-Diaz et al., 2008). Interestingly, of 621 mis-regulated genes in the pic1 mutant, 67 were also found to be misregulated in response to Pi starvation (Misson et al., 2005). This further supports our data and points toward a role for SWR1-dependent H2A.Z deposition in the modulation of diverse PSRs in Arabidopsis.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (Arabidopsis thaliana) ecotype Columbia-0, as well as arp6-1 and arp6-2 in the Columbia-0 background (Deal et al., 2005), were used in this study. Seeds were surface sterilized and grown under a 16/8-h photoperiod at 22°C with an average photosynthetically active radiation of 60 μmol m⁻² s⁻¹ provided by fluorescent tubes. For the ChiP experiment comparing Pi-replete and Pi-starved wild type, seedlings were grown hydroponically in a modified Murashige and Skoog (MS) medium containing 1.25 mM KH₂PO₄, pH 5.7, and 1.5% (w/v) Suc for 12 d and were transferred to fresh medium or Pi-deficient medium, which was the modified MS medium containing K₂SO₄ substituted for KH₂PO₄, for an additional 2 d. For all other experiments, seedlings were grown vertically in plates containing solid medium. Seeds were first germinated on plates containing half-strength MS medium, 1.2% (w/v) agar (A-1296, Sigma-Aldrich), and 1.5% (w/v) Suc for 5 d. Uniformly grown seedlings (with primary root length in the range of 1.5–2.5 cm) were transferred to the modified MS medium containing 1.25 mM KH₂PO₄, pH 5.7, and 1.5% (w/v) Suc and were grown for an additional 2 or 7 d, as stated. These are referred to as 7- and 12-d-old seedlings. For Pi-deficient medium, seedlings were transferred to a modified MS medium containing K₂SO₄ substituted for KH₂PO₄.

Seeding Morphological Analysis

For root hair analysis, images of the root hairs growing in the 5-mm section from the tip of the primary root of 7-d-old seedlings were captured using a stereomicroscope (Nikon SMZ-U). For documentation of primary root length, 12-d-old seedlings were scanned in a transmissive mode directly from the petri plates using a desktop scanner (UMAX Powerlook 2100 XL), and ImageJ (a Java image-processing program; http://rsb.info.nih.gov/ij) was used for measurement.

Soluble Pi Content

Shoots and roots were separated, rinsed in distilled water, blotted dry, frozen, and ground to a fine powder in liquid nitrogen. Approximately 20 to 25 mg of the ground tissue was suspended in 250 mL of 1% glacial acetic acid, vortexed, and centrifuged to pellet the cellular debris. The supernatant was assayed for Pi using a phosphomolybdate colorimetric assay (Ams, 1966).

Acid Phosphatase Activity

Shoots from 12-d-old seedlings were excised and ground to a powder in liquid nitrogen. Between 40 and 50 mg of frozen powder was used for the assays. Acid phosphatase activity was quantified by a p-nitrophenyl phosphosphate hydrolysis assay based on spectrophotometric measurement at 405 nm (Richardson et al., 2001) and is expressed as μU/mg protein. Total protein was estimated separately using Bradford’s reagent.

Starch Accumulation Analysis

Leaves from 12-d-old seedlings were excised, soaked in 95% ethanol overnight, and rinsed twice with water. Accumulation of starch was visualized by staining with iodine solution. The iodine solution was made by combining 500 μL of 300 mM KI (final concentration 6 mM) and 500 μL of 0.1 M iodine (Aldrich iodine volumetric standard) to a final volume of 10 mL. The tissues were stained for 5 min and rinsed with water. Images were captured under the stereomicroscope.

Quantitative Reverse Transcription-PCR

Twelve-day-old seedlings were separated into roots and shoots. Total RNA was isolated using the Qiagen RNeasy plant mini kit. RNA was treated with RNase-free DNase I (Promega) according to the manufacturer’s instructions. One microgram of RNA was reverse transcribed with the SuperScript III first-strand synthesis kit (Invitrogen). Real-time PCR was performed with an Applied Biosystems 7500 real-time PCR system using SYBR Green detection chemistry (Applied Biosystems) and gene-specific primers. The 40S ribosomal protein S16 gene (RPS16A; At2g09990) was used as an internal control, and the relative expression levels of the genes were computed by the 2⁻ΔΔCt method of relative quantification (Livak and Schmittgen, 2001). Oligonucleotide primer sequences are available upon request.

ChiP

ChiP was performed as described (Gendrel et al., 2005). For each experiment, 1 to 2 g of tissue (12-d-old shoots or roots or 14-d-old hydroponically grown whole seedlings) was used. The affinity-purified H2A.Z antibody was

LITERATURE CITED


