Phosphate Starvation Root Architecture and Anthocyanin Accumulation Responses Are Modulated by the Gibberellin-DELLA Signaling Pathway in Arabidopsis[1[OA]]

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Phosphate (Pi) is a macronutrient that is essential for plant growth and development. However, the low mobility of Pi impedes uptake, thus reducing availability. Accordingly, plants have developed physiological strategies to cope with low Pi availability. Here, we report that the characteristic *Arabidopsis thaliana* Pi starvation responses are in part dependent on the activity of the nuclear growth-repressing DELLA proteins (DELLAs), core components of the gibberellin (GA)-signaling pathway. We first show that multiple shoot and root Pi starvation responses can be repressed by exogenous GA or by mutations conferring a substantial reduction in DELLA function. In contrast, mutants having enhanced DELLA function exhibit enhanced Pi starvation responses. We also show that Pi deficiency promotes the accumulation of a green fluorescent protein-tagged DELLA (GFP-RGA [repressor of *ga1-3*]) in root cell nuclei. In further experiments, we show that Pi starvation causes a decrease in the level of bioactive GA and associated changes in the levels of gene transcripts encoding enzymes of GA metabolism. Finally, we show that the GA-DELLA system regulates the increased root hair length that is characteristic of Pi starvation. In conclusion, our results indicate that DELLA-mediated signaling contributes to the anthocyanin accumulation and root architecture changes characteristic of Pi starvation responses, but do not regulate Pi starvation-induced changes in Pi uptake efficiency or the accumulation of selected Pi starvation-responsive gene transcripts. Pi starvation causes a reduction in bioactive GA level, which, in turn, causes DELLA accumulation, thus modulating several adaptively significant plant Pi starvation responses.

Phosphate (Pi) is an essential plant macronutrient (Raghothama, 1999). Phosphorus forms insoluble compounds in acid soils or is unevenly distributed as Pi in alkaline soils (Holford, 1997). Thus, although the total amount of phosphorus is high in many soils, Pi availability is often a significant limiting factor for plant growth in both natural and agricultural systems (López-Bucio et al., 2000). To sustain growth in such limiting conditions, plants have evolved a number of developmental and metabolic responses to adapt both the internal Pi status in planta and the external soil Pi availability. These responses include changes in root morphology and architecture, accumulation of anthocyanin, and increases in the synthesis and secretion of organic acids into the rhizosphere (which enhance the utilization of Pi from insoluble inorganic compounds; del Pozo, 1999; Raghothama, 1999; López-Bucio et al., 2002). One characteristic of plant Pi starvation response is simultaneous reduction in shoot growth and increase in root proliferation. The outcome of this response is the formation of a highly branched root system (associated with reduced primary root length, increased lateral root number, and density) and increases in both frequency and length of root hairs. These changes enhance the exploratory capacity of roots to search for Pi-rich patches present in the soil (Raghothama, 1999; Lynch and Brown, 2001). Recently, *LPR1* (for *LOW PHOSPHATE ROOT1*), a major quantitative trait locus with a large effect on primary root growth arrest in response to Pi starvation, has been isolated (Svistoonoff et al., 2007). Loss-of-function mutations of *LPR1* and its close paralog, *LPR2*, both of which encode multicopper oxidases, reduce Pi starvation-induced inhibition of primary root growth. This suggests that LPR1 protein enables cells of the Arabidopsis (*Arabidopsis thaliana*) primary root cap to sense low-phosphate (LP) conditions, thus triggering root growth arrest (Svistoonoff et al., 2007).

The phytohormone auxin and the associated polar auxin transport mechanism are known to be essential for lateral root formation (Muday and Haworth, 1994; Reed et al., 1998; Casimiro et al., 2001). When plants

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are grown in LP conditions, exogenous auxin treatment dramatically inhibits the growth of primary roots and induces the formation of lateral roots. In contrast, a 10- to 100-fold higher auxin dose is necessary for seedlings grown in high-Pi (HP) conditions to develop a similar root architecture to that induced by Pi starvation (López-Bucio et al., 2002). In addition, auxin-resistant mutants *axr2-1*, *axr3-1*, and a*xr4-1* display normal responses to Pi deficiency, whereas the *iaa28-1* mutant displays resistance to the stimulatory effects of LP on root hair and lateral root formation (López-Bucio et al., 2002). These observations suggest that Pi starvation increases the sensitivity of Arabidopsis roots to auxin (López-Bucio et al., 2002).

Additional phytohormones are also known to be involved in inhibiting primary root growth and in promoting the production of both lateral roots and root hairs of Pi-starved Arabidopsis seedlings (Gilbert et al., 2000; Skene and James, 2000; López-Bucio et al., 2002; Al-Ghazi et al., 2003). For example, exogenous cytokinin treatment reduces the expression of selected Pi starvation-responsive marker genes (e.g. *At4*, *AtIPS1*, *AtPT1*, and *AtACP5*; Muchhal et al., 1996; del Pozo et al., 1999; Martin et al., 2000). Recently, it has been shown that cytokinin modulates the level of meristem cell cycle activity and that this, in turn, influences the expression of Pi starvation-responsive genes (Lai, et al., 2007). Ethylene is also involved in primary root elongation and root hair formation of seedlings grown in Pi starvation conditions (Ma et al., 2003; He et al., 2005). However, analysis of the root architecture of ethylene-signaling mutants, such as *etr1*, *ctr1*, *ein2*, *ein3*, and *hls1*, and of plants treated with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid show that ethylene does not promote the formation of lateral roots when Pi is limiting (López-Bucio et al., 2002).

The role of the phytohormone GA in Pi starvation responses remains largely unknown. Accordingly, we performed experiments to determine whether the GA-DELLA growth regulatory system contributes to Pi starvation plant growth responses. GA plays an important role in regulating plant growth and development throughout the life cycle, including seed germination, root elongation, hypocotyl growth, leaf expansion, floral initiation, and floral development (Hooley, 1994; Richards et al., 2001). GA-deficient mutant plants, such as the Arabidopsis *ga1-3* mutant, are dwarfed, exhibit dark-green leaves, and are late flowering, whereas exogenous GA treatment can restore normal growth to these mutants (Koornneef and van der Veen, 1980). GA promotes plant growth by relieving the growth restraint imposed by a family of nuclear growth-repressing DELLA proteins (DELLAs; Peng et al., 1997; Silverstone et al., 1998; Dill and Sun, 2001; King et al., 2001; Lee et al., 2002; Wen and Chang, 2002; Fu and Harberd, 2003). In Arabidopsis, the DELLAs comprise a family of five distinct proteins (GAI, RGA [repressor of *ga1-3*], RGL1, RGL2, and RGL3; Lee et al., 2002). Binding of bioactive GA to the Arabidopsis GA receptors (AtGID1a, AtGID1b, and AtGID1c) promotes interaction between these GA receptors and DELLAs (Nakajima et al., 2006). The DELLAs are subsequently polyubiquitinated by the $SCF^{SLY1/SLY2}$ E3 ubiquitin ligase and thus targeted for destruction in the 26S proteasome (Fu et al., 2002; McGinnis et al., 2003; Sasaki et al., 2003; Dill et al., 2004; Fu et al., 2004; Griffiths et al., 2006). Thus, DELLAs restrain plant growth, whereas GA stimulates growth by promoting destruction of the DELLAs.

Recent advances have revealed that the DELLAs play important roles in many aspects of the adaptation of plant growth and development in response to environmental variables (Fu et al., 2002; Lee et al., 2002; Fu and Harberd, 2003; Yu et al., 2004; Cao et al., 2005; Achard et al., 2006, 2007a, 2007b; Penfield et al., 2006). In this study, we systematically determined whether the GA-DELLA signaling pathway contributes to the control of plant Pi starvation responses. We found that exogenous GA overcomes several of the characteristic growth responses of Arabidopsis to Pi starvation and that mutants that are substantially DELLA deficient do not exhibit these Pi starvation growth responses. We further found that Pi starvation promotes the accumulation of a GFP-tagged DELLA (GFP-RGA) in root cell nuclei. Thus Pi starvation growth responses are, in part, determined by DELLA accumulation. However, we also found that Pi starvation does not alter the GA sensitivity of 26S proteasome-dependent DELLA destruction. This latter observation suggested that Pi starvation might cause the accumulation of DELLAs by reducing the levels of bioactive GA. Accordingly, we found that Pi starvation induced changes in the levels of transcripts encoding GA metabolism enzymes and decreases in bioactive GA levels. We additionally found that the GA deficiency *ga1-3* mutation confers a significant reduction in root hair length in Pi-starved plants and that GA treatment can restore this root hair length to that of wild type. Thus, an appropriate bioactive GA level is necessary for the increased root hair growth that is characteristic of Pi-starved roots. We conclude that DELLA restraint is a component of the mechanism via which Pi starvation modulates growth. Essentially, Pi starvation reduces bioactive GA levels, thus causing the accumulation of DELLAs and, in turn, triggering multiple Pi starvation responses, including alteration of root architecture, reduction of shoot growth, and accumulation of anthocyanin.

RESULTS

Pi Starvation Regulates Root Growth and Architecture via a GA-Dependent Mechanism

Inhibition of primary root growth is a characteristic plant Pi starvation response (Lynch and Brown, 2001; López-Bucio et al., 2003). For example, Arabidopsis seedling primary roots grow longer in HP (1 mM NaH_2PO_4) than in LP (10 μM NaH_2PO4) conditions (Fig. 1A). Primary root length is also regulated by GA. The length

of the primary seedling root of the GA-deficient *ga1-3* mutant is shorter than that of wild type, whereas exogenous GA can restore *ga1-3* primary roots to wild-type length (Fu and Harberd, 2003). To determine the effect of GA on the seedling primary root Pi starvation response, we compared the length of primary roots, numbers of lateral roots, and lateral root densities of wild-type (Columbia) and GA-deficient *ga1-t* mutant seedlings grown in HP versus LP conditions. We found that GA increases the primary root length of wild-type seedlings grown in LP (Fig. 1, B and C). In addition, LP-grown *ga1-t* primary root length was 2- to 3-fold reduced compared with that of wild type, whereas exogenous GA restored the LP-grown *ga1-t* primary root to wild-type (+GA) length (Fig. 1, B and D). There was no detectable difference in the root architectures of GA-treated and control wild-type seedlings grown in HP (Fig. 1D). Thus, GA increases the primary root length of wild-type seedlings grown in LP.

Pi starvation promotes the growth of lateral roots (López-Bucio et al., 2002; Fig. 1, A–C). We found that exogenous GA did not significantly alter the number of lateral roots formed by wild-type seedlings in HP or LP (Fig. 1E). However, due to GA-promoted increases in primary root length, exogenous GA caused a reduction in lateral root density in LP-grown wild-type seedlings (Fig. 1F). In HP, at 12 d following transfer, wild-type seedlings have a long primary root and relatively few lateral and secondary lateral roots. However, LP-grown wild-type seedlings produce a highly branched root system with abundant lateral and secondary lateral roots (Fig. 1G). Moreover, LP-grown *ga1-t* mutant seedlings produce even higher numbers of secondary lateral roots than do LP-grown wild-type seedlings (Fig. 1, B and G). Interestingly, we found that exogenous GA significantly reduced the number of secondary lateral roots (in both wild type and *ga1-t*) of seedlings grown in LP (Fig. 1G).

An increased root-to-shoot ratio is another characteristic plant Pi starvation response (Lynch and Brown, 2001; López-Bucio et al., 2003; Fig. 1H). We found that exogenous GA decreases the root-to-shoot ratio of LP-grown wild-type and *ga1-t* plants (Fig. 1H). Thus, exogenous GA promotes the growth of both roots and shoots in LP, suggesting the possibility that Pi starvation reduces growth via a reduction in bioactive GA levels.

Taken together, the above results indicate that GA contributes (at least partially) to regulating the alterations in root and shoot growth and architecture that are characteristic of plant Pi starvation responses and play a particularly prominent role in controlling the development of secondary lateral roots.

Pi Starvation Inhibits Plant Growth via the GA-DELLA Signaling Mechanism

DELLAs act as plant growth repressors in GA signaling (Richards et al., 2001). GA promotes growth by targeting the growth-restraining DELLAs for destruction in the 26S proteasome (Fu and Harberd, 2003; McGinnis et al., 2003; Dill et al., 2004; Fu et al., 2004). To determine whether GA regulates plant Pi starvation responses via the GA-DELLA mechanism, we examined the Pi starvation responses of wild-type (Landsberg *erecta*), *ga1-3*, *gai-t6 rga-t2 rgl1-1 rgl2-1*, *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1*, *gai*, and *sly1-10* mutant seedlings. Mutants carrying the *gai-t6*, *rga-t2*, *rgl1-1*, and *rgl2-1* mutations, respectively, lack the DELLAs GAI, RGA, RGL1, and RGL2 (although they retain RGL3) and are thus substantially DELLA deficient (Cheng et al., 2004; Cao et al., 2005; Achard et al., 2006). *gai* and *sly1-10* confer GA insensitivity because they reduce the susceptibility of DELLAs to GA-promoted degradation, thus enhancing DELLA accumulation. We found that the primary root length of LP-grown *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* mutant seedlings was approximately twice that of LP-grown control GA-deficient *ga1-3* mutant seedlings (Fig. 2A). Furthermore, the primary root length of LP-grown *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* mutant seedlings was identical to that of GA-treated LP-grown wild-type, *ga1-3*, and *gai-t6 rga-t2 rgl1-1 rgl2-1* seedlings. Conversely, the LP-grown primary root lengths of the GA-insensitive *gai* and *sly1-10* mutant lines were unaffected by exogenous GA (Fig. 2A). In addition, when wild-type seedlings were grown in LP, the root length was only 28% of that grown in HP and the LP-to-HP growth-inhibition ratio was therefore approximately 72% in the absence of GA (Fig. 2B). But GA treatment restored root growth in LP conditions and the wild-type LP-to-HP growth inhibition ratio was reduced to approximately 63% (Fig. 2B). These GA-promoted differentials were enhanced in *ga1-3* and reduced in DELLA-deficient lines (*gai-t6 rga-t2 rgl1-1 rgl2-1*; *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1*) and in GA-insensitive *gai* and *sly1-10* mutant lines (Fig. 2B).

We also found, similarly to what had been observed for root growth, that the effects of Pi starvation on shoot growth (plant height) are mediated in part via the GA-DELLA pathway. For example, either exogenous GA or substantial DELLA deficiency (conferred by *gai-t6 rga-t2 rgl1-1 rgl2-1*) increases the stem length of LP-grown wild-type plants (Fig. 2, C and D). GA increases the stem length of LP-grown *ga1-3* plants, but has no effect on LP-grown *ga1-3* plants that are substantially DELLA deficient (*ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1*) or on LP-grown *gai* plants (Fig. 2, C and D). Taken together, the above results indicate that Pi starvation affects both root and shoot via a mechanism that is (at least in part) dependent on the GA-DELLA signaling system.

Pi Starvation Induces Anthocyanin Accumulation via a DELLA-Dependent Mechanism

The visible accumulation of anthocyanin pigmentation is one of the characteristic responses of plants to Pi starvation. Anthocyanins and other polyphenolic

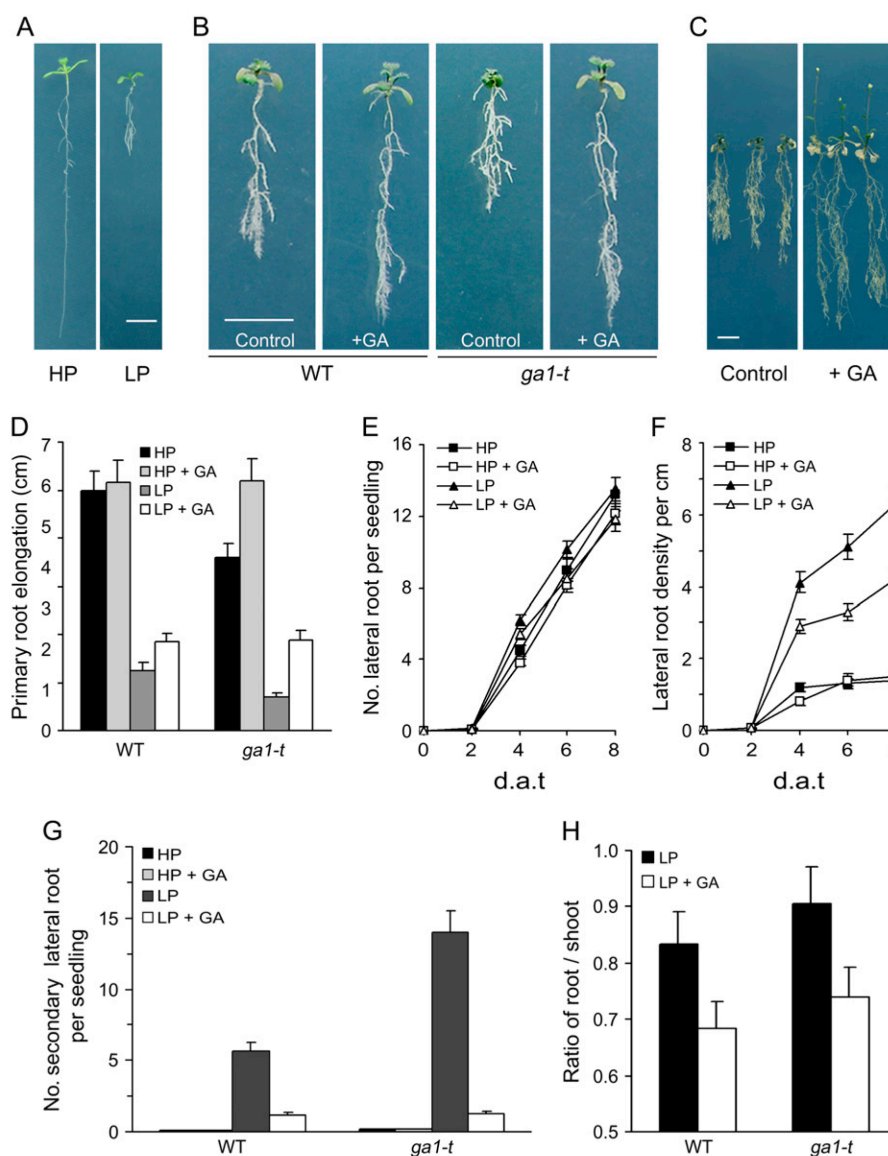

Figure 1. GA promotes root growth and changes in root morphology in responses to Pi starvation. Four-day-old Arabidopsis Columbia wild-type (WT) and GA-deficient *ga1-t* mutant seedlings were transferred to LP or HP conditions (as described in "Materials and Methods") in the presence or absence of exogenous 1 μm GA. A, Pi starvation affects primary root growth. Four-day-old wild-type seedlings were transferred to LP or HP medium for 6 d. B and C, Effects of GA treatment on root growth and morphology of seedlings grown in LP conditions for 8 d (B) and 28 d (C). D, Comparison of primary root elongation of 8-d-after-transfer (d.a.t.) wild-type and *ga1-t* mutant seedlings grown in LP and HP conditions in the presence or absence of GA. E and F, Kinetic analysis of lateral root number per each seedling (E) and lateral root density (F) of wild-type seedlings grown during Pi starvation. G, Effects of GA and Pi starvation on secondary lateral root development of 12-d-after-transfer wild-type and *ga1-t* seedlings. H, Root-to-shoot ratio of wild-type and *ga1-t* mutant seedlings grown in Pi starvation conditions. Results are presented as means with error bars representing SE. Bar = 1 cm.

compounds (e.g. flavonols and condensed tannins) have a wide range of functions in plants related to UV absorption, pathogen attack, and nutrient stress (Stewart et al., 2001; Kliebenstein, 2004). To gain insight into the relationship between DELLA function and anthocyanin accumulation, we compared the effects of Pi starvation on the anthocyanin content of wild-type and *gai-t6 rga-t2 rgl1-1 rgl2-1* seedlings. In the absence of exogenous GA, leaves of LP-grown wild-type plants were visibly purple and accumulated higher levels of anthocyanin than did HP-grown controls (Fig. 3, A and B). In contrast, LP-grown *gai-t6 rga-t2 rgl1-1 rgl2-1* plants were visibly less purple than LP-grown wild-type plants (Fig. 3A) and accumulated anthocyanin to a lower level (Fig. 3B). GA treatment caused a reduction in anthocyanin accumulation in wild-type plants grown in LP conditions, whereas anthocyanin accumulation of LP-grown *gai-t6 rga-t2 rgl1-1 rgl2-1* plants was less responsive to GA-induced reductions (Fig. 3B). These results suggest that DELLA activity promotes the accumulation of anthocyanin during Pi starvation.

Anthocyanin is a branch for synthesis of flavonols via flavonol synthase in the flavonoid pathway. The enzymes of each step for anthocyanin synthesis are required: chalcone synthase, chalcone isomerase, flavone-3-β-hydroxylase, dihydroflavonol-4-reductase, leucoanthocyanidin dioxygenase (LDOX; Jaffe et al., 2006). Moreover, UDP-Glc-flavonoid 3-*O*-glucosyltransferase (UF3GT), UDP-glycose:UF3GT is a specific enzyme for anthocyanin synthesis (Jaffe et al., 2006). We next examined the effects of Pi starvation on the expression of genes encoding enzymes of anthocyanin metabolism. We found that wild-type plants grown in LP conditions accumulated relatively high levels of *PAP1* (for *PRODUCTION OF ANTHOCYANIN PIGMENT1*, *AtMYB75*; Borevitz et al., 2000), *F3′H* (flavone 3′ hydroxylase),

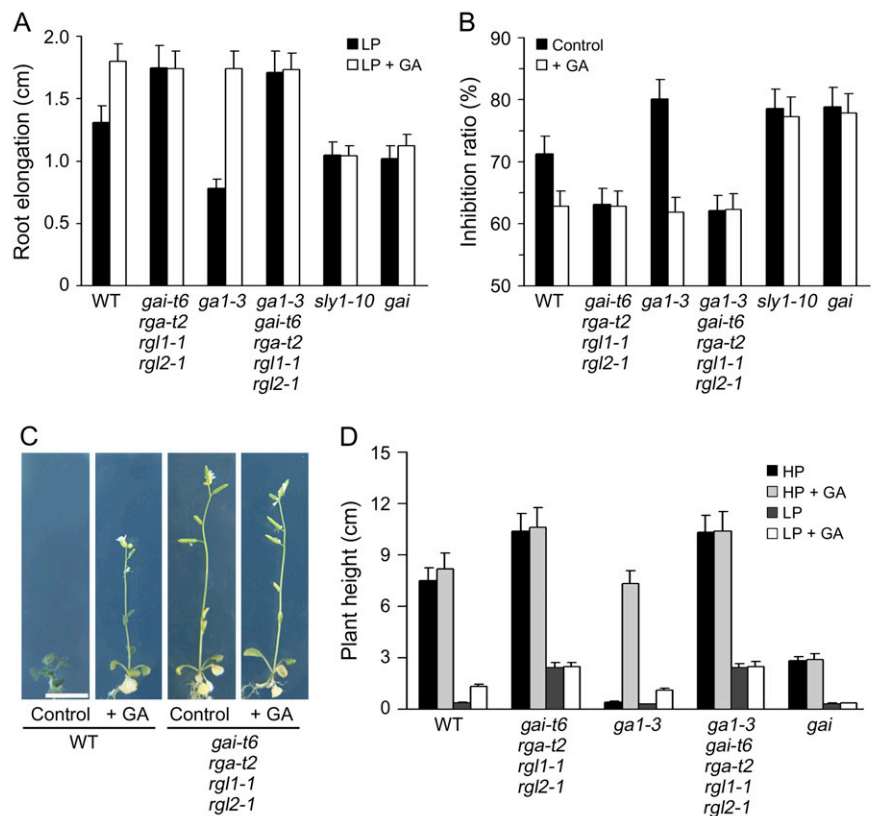

Figure 2. Pi starvation inhibits plant growth via a DELLA-dependent mechanism. A, Comparison of root growth of wild-type and DELLA-associated mutant seedlings. Four-day-old seedlings were transferred to LP conditions in the presence or absence of 1 μm GA for 6 d. B, Primary root growth inhibition ratios (1-LP/HP elongation length) from 4-d-old seedlings transferred to appropriate Pi conditions in the presence or absence of 1 μm GA for 6 d. C and D, Comparison of the plant heights of 4-d-old seedlings transferred to HP or LP conditions in the presence or absence of GA for 21 d. Results are presented as means with error bars representing SE. Bar = 1 cm.

LDOX, and *UF3GT* transcripts, and that GA treatment dramatically reduced the levels of these transcripts (Fig. 3C). These observations indicate that the anthocyanin accumulation characteristic of Pi starvation is due to increases in PAP1, F3′H, LDOX, and UF3GT activity, whereas the GA-promoted reduction in anthocyanin level is the consequence of a reduction in PAP1, F3′H, LDOX, and UF3GT activity. Furthermore, LP-grown *gai-t6 rga-t2 rgl1-1 rgl2-1* plants contained undetectable levels of *F3′H* and *LDOX* transcripts in both HP and LP conditions and had significantly lower than LP-grown wild-type plant levels of *UF3GT* transcripts (Fig. 3C). Interestingly, exogenous GA promoted a decrease in the levels of both *UF3GT* and *PAP1* transcripts in both wild type and *gai-t6 rga-t2 rgl1-1 rgl2-1*, whereas there were no differences in levels of *PAP1* transcripts between LP-grown wild-type and *gai-t6 rga-t2 rgl1-1 rgl2-1* plants (regardless of the presence or absence of exogenous GA; Fig. 3C). These observations indicate that down-regulation of *PAP1* transcript level is GA dependent, but not dependent on the function of GAI, RGA, RGL1, or RGL2.

In conclusion, the data in Figure 3 indicate that the accumulation of anthocyanin that is characteristic of Pi starvation is the consequence of increases in expression of genes of anthocyanin metabolism. For some of these genes (*F3′H* and *LDOX*), this up-regulated expression is GA-DELLA dependent. For others (*UF3GT* and *PAP1*), GA can overcome LP-induced increases in transcript level via a mechanism that is independent of the DELLAs GAI, RGA, RGL1, and RGL2 (and may therefore be dependent upon RGL3 activity).

DELLAs Do Not Detectably Regulate LP-Induced Changes in Pi Uptake Efficiency or Levels of Pi Starvation-Induced Transcripts

Plants have evolved numerous adaptive responses to Pi starvation, including changes in root architecture, accumulation of anthocyanin pigments, and improvement of Pi uptake efficiency (Raghothama, 1999). Activation of expression of plant Pi starvation marker genes, such as genes encoding Pi transporters, appears to be a universal response, suggesting the presence of a highly regulated molecular network controlling the expression of the genes involved (Rausch and Bucher, 2002). The above-described experiments had indicated a link between GA signaling and various aspects of the plant Pi starvation response. It remained possible that the growth promoted by GA under Pi starvation was due to increases in Pi transporter levels and consequent enhancement of Pi uptake. We therefore analyzed the effect of GA and DELLAs on the expression of Pi starvation-induced marker genes. As shown previously, we found that the expression of genes encoding Pi transporters (*AtPT1* and *AtPT2*) and of additional Pi starvation-responsive marker genes (*AtACP5*, *At4*, and *AtIPS1*) was enhanced in LP conditions (Muchhal et al.,

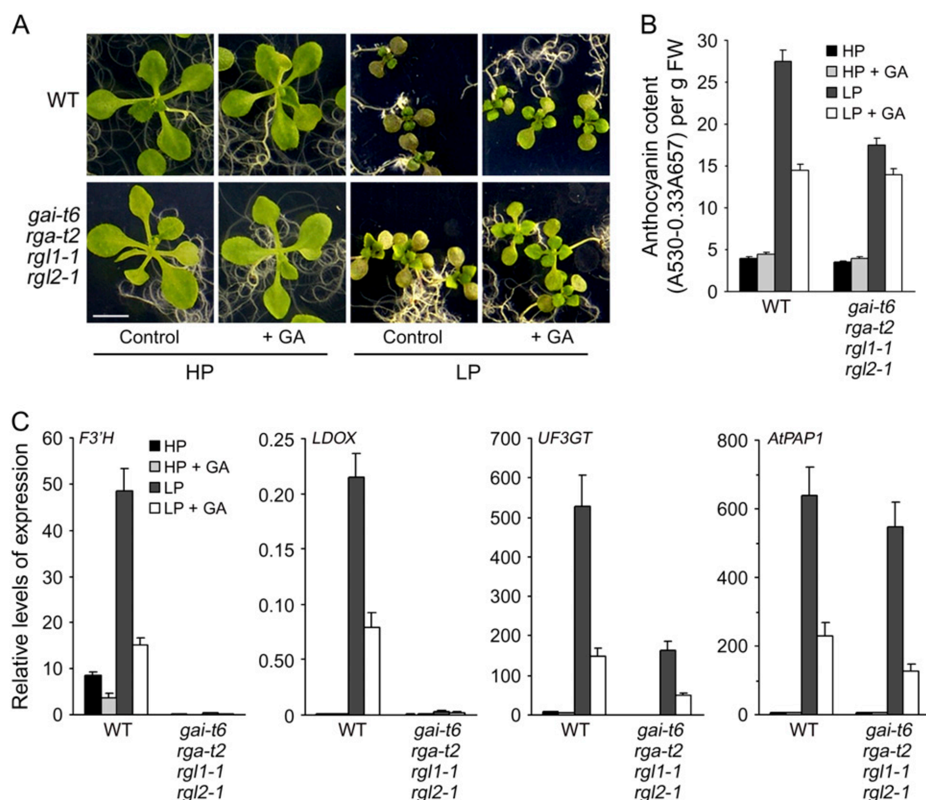

Figure 3. DELLAs contribute to changes in anthocyanin accumulation and levels of transcripts encoding enzymes of anthocyanin metabolism in Pi starvation conditions. A, Effects of Pi availability and GA treatment on anthocyanin accumulation for 12-d-after-transfer Arabidopsis seedlings. B, Comparison of anthocyanin content of wild-type and *gai-t6 rga-t2 rgl1-1 rgl2-1* mutant seedlings as shown in A. C, Levels of *F3′H*, *PAP1*, *LDOX*, and *UF3GT* gene transcripts in seedlings as shown in A and B (determined by real-time PCR). Results are displayed as the copy per 1,000 copy of 18S rRNA.

1996; del Pozo et al., 1999; Martin et al., 2000; Fig. 4A). However, GA treatment had no detectable effect on the levels of these marker gene transcripts in wild-type plants (Fig. 4A). Furthermore, mutant lines having altered GA-DELLA signaling functions (e.g. *gai-t6 rga-t2 rgl1-1 rgl2-1* and *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1*) also exhibited LP-induced accumulation of transcripts of the above Pi starvation-responsive marker genes, accumulations that were not changed by exogenous GA (Fig. 4A). Thus, the growth promotion of GA-treated LP-grown wild-type seedlings (or of LP-grown seedlings substantially deficient for DELLA function) is unlikely to be due to a DELLA-dependent further increase in Pi uptake efficiency (resulting from an increase in the levels of transcripts encoding the Pi transporters AtPT1 and AtPT2).

We next compared the phosphorus content of wild-type and *gai-t6 rga-t2 rgl1-1 rgl2-1* seedlings grown in HP versus LP conditions. Whereas plants grown in HP contained more phosphorus than plants grown in LP, we found no significant differences attributable to genotype (the phosphorus content of wild-type and *gai-t6 rga-t2 rgl1-1 rgl2-1* seedlings were identical in HP and again in LP; Fig. 4B). In addition, GA treatment had no detectable effect on phosphorus content (Fig. 4B) or on the content of additional nutrients such as manganese, iron, zinc, calcium, and magnesium (Fig. 4C). Thus, DELLAs do not detectably alter changes in Pi absorption or the expression of Pi starvation-responsive marker genes.

Pi Starvation Regulates the Levels of Transcripts Encoding Enzymes of GA Metabolism

Because Pi starvation inhibits plant growth via a mechanism that is in part DELLA dependent (as shown in Figs. 1 and 2), we determined whether DELLA-dependent Pi starvation-induced inhibition of root growth is associated with DELLA accumulation. These experiments used a transgenic line expressing a *pRGA*:*GFP-RGA* construct (that encodes a fusion protein comprising the GFP and the DELLA protein RGA; Silverstone et al., 2001). We found that fluorescence attributable to GFP-RGA was more intense in root cell nuclei of *pRGA*:*GFP-RGA* seedlings grown in LP conditions than it was in HP-grown *pRGA*:*GFP-RGA* seedlings (Fig. 5A). Furthermore, immunologically detectable GFP-RGA (detected using an anti-GFP antibody) was more abundant in LP-grown roots than in HP-grown roots (Fig. 5B). These observations indicate that Pi starvation increases the accumulation of RGA in root cell nuclei. The GFP-RGA fusion protein is rapidly destroyed following GA treatment when *pRGA*:*GFP-RGA* seedlings are grown in HP conditions (Silverstone et al., 2001; Fu and Harberd, 2003; Fig. 5, A and B). In contrast, GFP-RGA was clearly detectable in root cell nuclei of LP-grown *pRGA*:*GFP-RGA* seedlings after 1.5 h GA treatment (and not after 1.5 h GA treatment of HP-grown *pRGA*:*GFP-RGA* seedlings [Fig. 5A]). However, GFP-RGA was not detectable in root cell nuclei of both LP- and HP-grown *pRGA*:*GFP-RGA* seedlings within

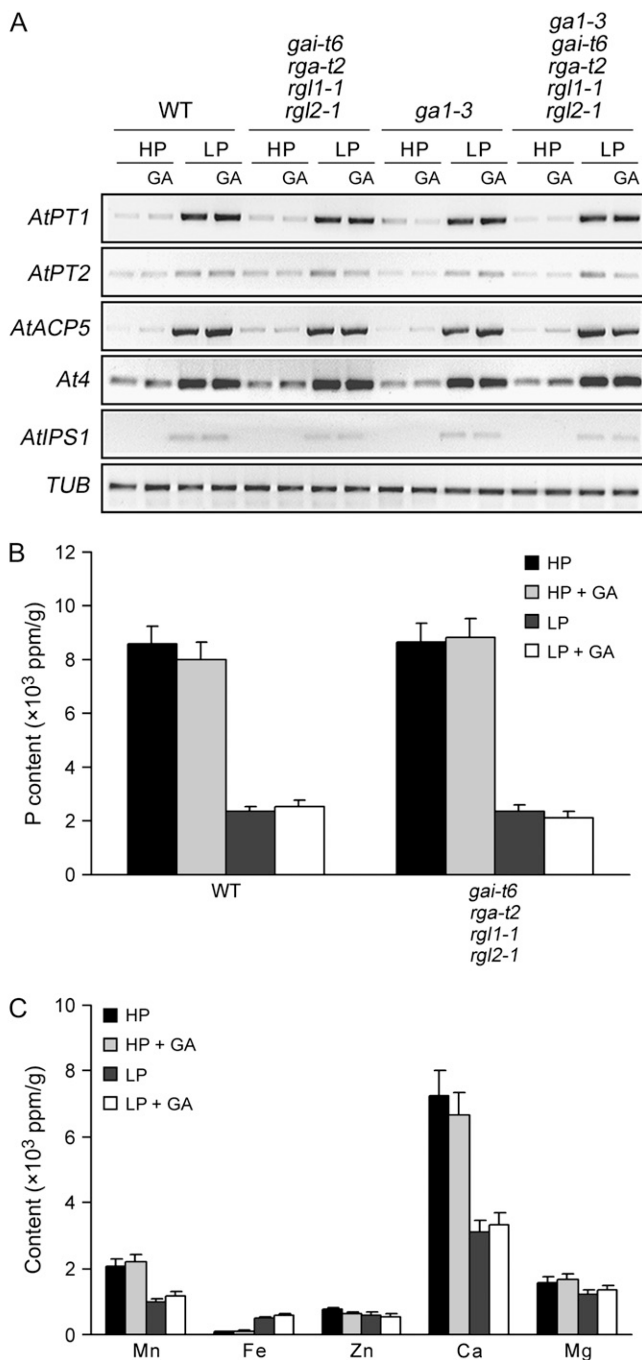

Figure 4. DELLAs do not contribute to the expression of Pi starvation-induced marker genes or to the regulation of phosphorus absorption. A, Comparison of the levels of Pi starvation-induced marker gene transcripts (determined by RT-PCR). Plants were 6-d-after-transfer wild-type, *gai-t6 rga-t2 rgl1-1 rgl2-1*, *ga1-3*, and *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* mutant seedlings grown with treatments as indicated. *Tubulin* (*TUB*) transcripts provided loading control. B and C, Comparison of the phosphorus and other micronutrient contents of 6-d-after-transfer wild-type, *gai-t6 rga-t2 rgl1-1 rgl2-1*, *ga1-3*, and *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* mutant seedlings grown in the same conditions as in A. Results are presented as means with SE bars.

4 h of onset of GA treatment (Fig. 5, A and B). These observations suggest that Pi starvation enhances the accumulation of DELLAs, but does not change the vulnerability of DELLAs to GA-promoted destruction in the 26S proteasome.

We next determined whether the LP-promoted accumulation of GFP-RGA was associated with Pi starvation-induced increases in the levels of gene transcripts encoding DELLAs or decreases in the levels of gene transcripts encoding other GA-signaling components, such as the SLY1 components of the $SCF^{SLY1/SLY2}$ E3 ubiquitin ligase (Dill et al., 2004; Fu et al., 2004) and the three Arabidopsis GA receptors (AtGID1a, AtGID1b, and AtGID1c; Griffiths et al., 2006). We found that levels of *SLY1*, *RGA*, *GAI*, *RGL1*, *RGL2*, and *RGL3* transcripts were not affected by Pi status (Fig. 5C; data not shown). In contrast, whereas *AtGID1a* and *AtGID1b* transcript levels were not detectably different in LP or HP conditions, Pi starvation dramatically promoted *AtGID1c* transcript accumulation (Fig. 5C). Thus, whereas GFP-RGA accumulates in LP-grown *pRGA:GFP-RGA* root nuclei, this accumulation is unlikely to be due to increased accumulation of DELLA-encoding transcripts or to decreased accumulation of transcripts encoding the F-box (*SLY1*) and GA receptors (*AtGID1a*,*b*,*c*) of the GA-signaling pathway.

Whereas an increase in bioactive GA level causes a decrease in GFP-RGA accumulation, a reduction in bioactive GA level causes an increase in GFP-RGA accumulation (Silverstone et al., 2001; Fu and Harberd, 2003). We next analyzed whether the Pi starvation-induced accumulation of GFP-RGA might be the consequence of a decrease in bioactive GA level. Bioactive GA level is elevated by increases in the levels of transcripts encoding GA 20-oxidases (GA20ox) and GA 3-oxidases (GA3ox) or by decreases in the levels of transcripts encoding GA 2-oxidases (GA2ox; Chiang et al., 1995; Phillips et al., 1995; Thomas et al., 1999). We therefore analyzed the effects of Pi starvation on the levels of *GA20ox*, *GA3ox*, and *GA2ox* transcripts. We found evidence of reduced levels of *GA20ox1* transcripts in LP-grown (compared with HP-grown) seedlings whether determined via real-time reverse transcription (RT)-PCR (Fig. 5D) or visualized as the relative level of GUS activity expressed from a *pGA20ox1:GUS* (promoter-GUS) fusion construct (Fig. 5E). Although there was no detectable difference in *GA3ox1* transcript levels in shoots, roots were found to contain much lower levels of *GA3ox1* transcripts when grown in LP conditions than were found in HP conditions (Fig. 5C). In contrast, both shoots and roots of LP-grown seedlings had relatively high levels of *GA2ox2* transcripts (Fig. 5D).

Increases in the levels of *GA2ox2* transcripts (that encode an enzyme that deactivates bioactive GAs) and decreases in the levels of *GA20ox* and *GA3ox* transcripts (both of which encode enzymes of bioactive GA biosynthesis) would be expected to reduce the in planta levels of bioactive GAs. To test this possibility, we determined the levels of GA_4 (the principal bioactive GA

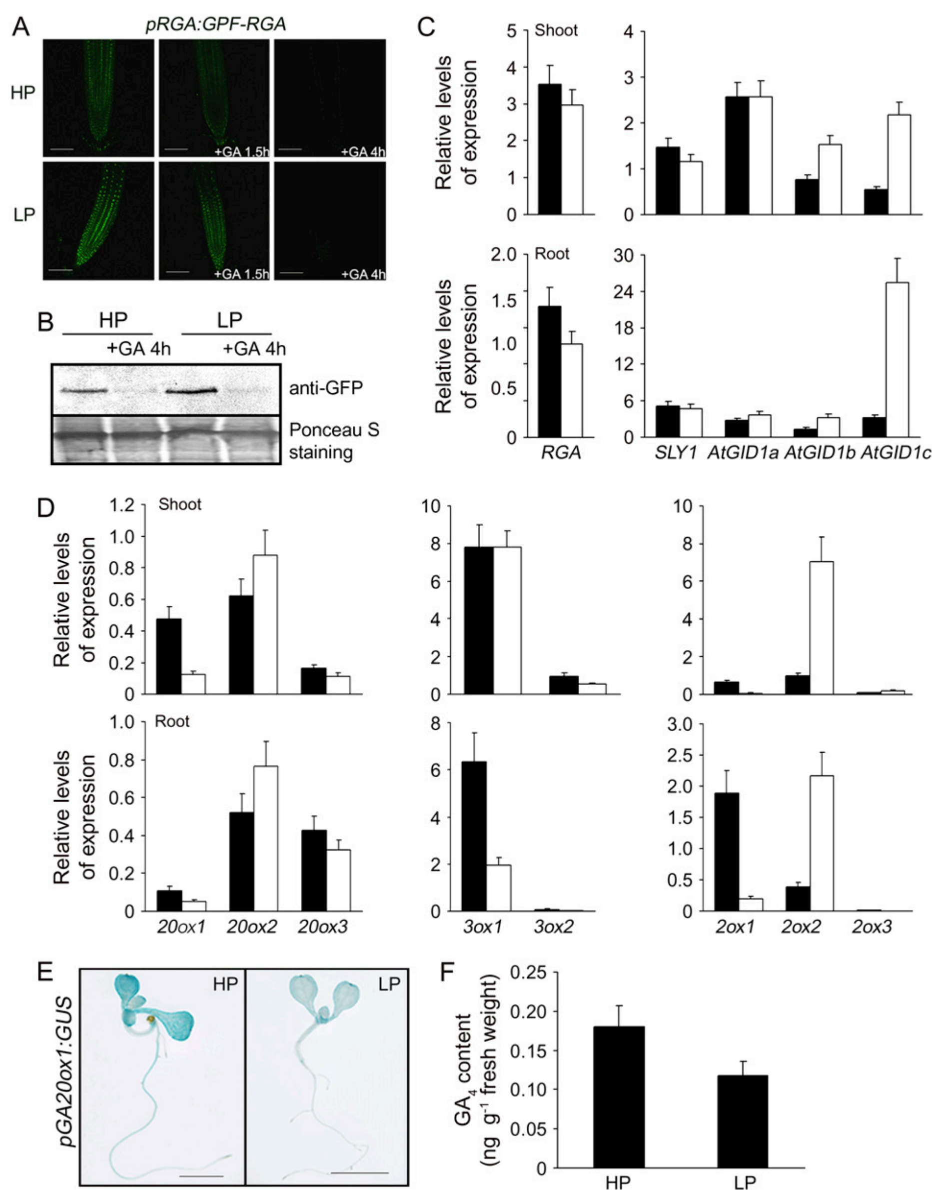

Figure 5. Pi starvation regulates the levels of GA biosynthesis gene transcripts. A, Pi starvation promotes accumulation of GFP-RGA protein in nuclei of 4-d-old *pRGA*:*GFP-RGA* seedlings grown on LP condition for 5 d, GFP-RGA fluorescence is still sensitive to GA response. B, Immunodetection of GFP-RGA in *pRGA*:*GFP-RGA* roots grown in HP or LP conditions in the presence or absence of GA. C, Levels of GA signaling *RGA*, *SLY1*, *AtGID1a*, *AtGID1b*, and *AtGID1c* gene transcripts (determined by real-time PCR) in 4-d-old seedlings grown on GM medium, then transferred to HP or LP conditions for 5 d. Results are displayed as the copy per 1,000 copy of 18S rRNA. D, Expression profiles of GA biosynthesis *GA 20-oxidase*, *GA 3-oxidase*, and *GA 2-oxidase* genes. Four-day-old wild-type seedlings were transferred to LP or HP conditions for 6 d, respectively, and then the samples were collected and analyzed using real-time PCR. Results are displayed as the ratio of expression to 18S rRNA. E, GUS staining of *pGA20OX1*:*GUS* seedling grown on GM medium, then transferred to LP condition for 5 d. F, Determination of GA_4 content of 4-d-old seedlings transferred to LP or HP conditions for 5 d using the gas chromatography-mass spectrometry method.

species in Arabidopsis) in wild-type seedlings grown in LP versus HP conditions. We found that the level of GA_4 in LP-grown seedlings was significantly less than was detected in HP-grown controls (Fig. 5F). Thus, Pi starvation causes a reduction in GA_4 levels and this reduction, in turn, likely explains the Pi starvation-induced accumulation of GFP-RGA.

Pi Starvation Enhancement of Root Hair Elongation Is GA Dependent

Growth of Arabidopsis seedlings in Pi-limiting conditions causes an increase in the length and frequency of root hairs, thus enlarging the root surface area and enhancing the ability of the roots to absorb phosphorus (Schikora and Schmidt, 2001; Ma et al., 2003; Fig. 3A). Recently, it has been reported that the phytohormones ethylene and auxin are involved in Pi starvation-induced root hair development in Arabidopsis (Schikora and Schmidt, 2001; He et al., 2005). To investigate the possible role of the GA-DELLA system in Pi deficiency-induced changes in the formation and growth of root hairs, we compared the roots of wild-type, *gai-t6 rga-t2 rgl1-1 rgl2-1*, *ga1-3*, and *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* seedlings. We found no significant differences in root hair formation in GA-treated and control LP-grown wild-type seedlings (Fig. 6A; data not shown), suggesting that DELLAs do not contribute to LP-stimulated changes in epidermal cell fate. Whereas the root hairs of LP-grown GA-deficient *ga1-3* mutants were mostly formed in the same position as in wild-type controls, the *ga1-3* root hair density was somewhat high, and

the lengths of individual *ga1-3* root hairs were much shorter than those of wild type (Fig. 6A). However, the length of LP-grown *ga1-3* root hairs could be restored to that of wild type by exogenous GA (Fig. 6, A and B). Thus, GA is required for Pi deficiency-induced root hair elongation. Pi starvation causes a reduction in GA_4 levels (Fig. 5F) and an increase in root *AtGID1c* transcript levels (Fig. 5C). Perhaps Pi starvation-enhanced AtGID1c GA receptor function explains the Pi starvation-induced root hair elongation. Furthermore, we found that LP-grown *gai-t6 rga-t2 rgl1-1 rgl2-1* root hairs are longer than those of LP-grown wild-type seedlings. The density of Pi-starved *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* root hairs was lower than that of *ga1-3* and slightly lower than that of the wild type (data not shown). These results indicate that GA-DELLA signaling contributes to the regulation of root hair length in Pi starvation conditions.

DISCUSSION

It has recently become apparent that the GA-DELLA mechanism plays an important role in modulating plant growth via integration of both environmental and endogenous signals (Lee at al., 2002; Fu and Harberd, 2003; Alvey and Harberd, 2005; Achard et al., 2006, 2007a, 2007b; Penfield et al., 2006). The work described in this article shows that the plant growth and developmental effects of nutrient limitation, in particular of Pi starvation, are also mediated (at least in part) via the GA-DELLA mechanism. First, we have shown that GA is involved in regulating Pi starvation-induced changes in root and shoot architecture and, in particular, in promoting the development of secondary lateral roots. Second, we have shown that Pi starvation inhibits plant growth and promotes anthocyanin accumulation and root hair elongation via mechanisms that are DELLA dependent. Third, we have shown that Pi starvation results in DELLA accumulation (accumulation of GFP-RGA) and that this accumulation is associated with a reduction in the levels of bioactive GA.

The mechanisms underlying Pi starvation signaling are well understood in bacteria and yeast (*Saccharomyces cerevisiae*; Torriani, 1990; Lenburg and O'Shea, 1996). However, it is not currently clear how plant primary responses to Pi starvation are initiated. Our results indicate that the plant GA-DELLA mechanism does not regulate the changes in Pi uptake efficiency or levels of Pi starvation-induced transcripts that are characteristic of Pi starvation. Thus, the GA-DELLA mechanism likely contributes to Pi starvation responses as follows. Following perception and initial signaling of Pi starvation conditions (via unknown mechanisms), the levels of gene transcripts encoding enzymes that activate bioactive GAs are reduced, whereas those that deactivate GAs are increased. In consequence, bioactive GA levels fall and DELLAs accumulate. Accumulation of DELLAs, in turn, contributes to a range of characteristic Pi starvation growth and developmental responses, including changes in shoot and root architecture, accumulation of anthocyanins, and root hair elongation.

Whereas our observations identify GA-DELLA-dependent components of the plant Pi starvation response, we have also identified DELLA-independent components. Thus, although a significant contributor, the GA-DELLA mechanism is not the sole developmental regulator of Pi starvation response. Future experiments will determine whether the developmental effects of nutrient limitation, in general, are partially DELLA dependent or whether DELLA dependency is specifically restricted to the Pi starvation response.

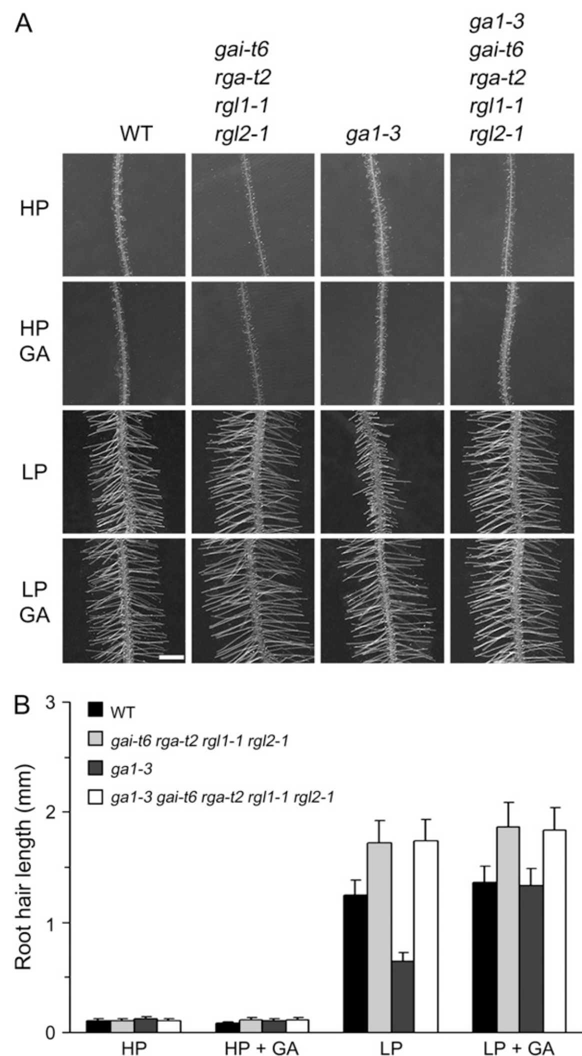

Figure 6. Bioactive GAs are required for Pi starvation promotion of root hair growth. A, Effect of Pi availability and GA treatment on root hair growth for 9-d-after-transfer wild-type, *gai-t6 rga-t2 rgl1-1 rgl2-1*, *ga1-3*, and *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* mutant seedlings. Bar = 1 mm. B, Comparison of root hair length in mature root hair zone of Arabidopsis seedlings shown in A; results are presented as means ± SE.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The experiments used transgenic line *pGA20ox1*:*GUS* and *ga1-t* Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia (laboratory strain genetic background). The Landsberg *erecta* laboratory strain, mutant lines of *ga1-3*, *gai*, *gai-t6 rga-t2 rgl1-1 rgl2-1*, *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1*, and *sly1-10* and transgenic line *pRGA*:*GFP-RGA* were as described previously (Reed et al., 1993; Whitelam et al., 1993; Cheng et al., 2004; Fu et al., 2004; Achard et al., 2006). All seeds were surface sterilized and placed on glucose minimal (GM) medium plates at 4°C for 4 d to synchronize germination as described previously (Fu and Harberd, 2003). Plates were then placed in vertical orientation in controlled-environment chambers (22°C, 16-h light). Four-day-old seedlings were transferred to LP medium (10 μM NaH_2PO4) or HP medium (1 mM NaH_2PO4), which was supplemented with 2.0 mM NH_4NO_3, 1.9 mM KNO_3, 0.3 mM $CaCl_2{\cdot}2H_2O$, 0.15 mM $MgSO_4{\cdot}7H_2O$, 5 μM KI, 100 μM H_3BO_3, 100 μM $MnSO_4{\cdot}H_2O$, 30 μM $ZnSO_4{\cdot}7H_2O$, 1 μM $Na_2MoO_4{\cdot}2H_2O$, 0.1 μM $CuSO_4{\cdot}5H_2O$, 0.1 μM $CoCl_2{\cdot}6H_2O$, 100 μM $FeSO_4{\cdot}7H_2O$, 100 μM $Na_2EDTA{\cdot}2H_2O$, and 1% Suc. Plants were maintained at 65 μM m^{-2} s^{-1} photosynthetically active radiation and placed in vertical orientation in controlled-environment chambers (22°C, 16-h light).

Root Growth Experiments

Arabidopsis root length, lateral roots, and root hairs were photographed with a Leica MZ16FA stereomicroscope with Leica IM50 software. Root tissues were scanned and length was measured using SigmaScan Pro 5 software (Systat Software Inc.). For each set of experiments, at least 60 seedlings were measured.

Detection of GFP Fluorescence and GUS Staining

For GUS staining, Arabidopsis seedlings were incubated for 4 h at 37°C (in 0.5 mg/mL of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide in 100 mM sodium phosphate, pH 7.0) and stained seedlings were cleared and photographed. Fluorescence due to GFP-RGA in root cell nuclei was determined by Olympus laser confocal microscopy as described previously (Fu and Harberd, 2003).

Detection of Pi and Anthocyanin

Seedlings were grown on one-half-strength Murashige and Skoog medium for 3 d. Subsequently, seedlings were transferred to HP or LP medium with or without GA treatment for 12 d. Fifty milligrams (fresh weight) of seedlings were collected and anthocyanin content was measured as described before (Kim et al., 2003). For measurement of Pi content, seedlings were transferred to LP or HP medium for 7 d, then collected and dried at 80°C for 48 h. Pi content of 50 mg of seedlings (dry weight) was evaluated by the vanadate-molybdate colorimetric method (Hesse, 1971).

Transcript Analysis

Total RNA was extracted using TRIzol reagent (Invitrogen). Semiquantitative RT-PCR was performed as previously described (Fu et al., 2004). Real-time PCR was performed using SYBR green PCR master mix (Applied Biosystems) in optical 96-well reaction plates (Applied Biosystems) on an Eppendorf mastercycler system. All reactions were repeated at least three times. The relative quantity was based on the comparative Ct (threshold cycle) method and α-tubulin as control.

Primer pairs used for PCR amplification were as follows: *GA20ox1*, 5′-aaatccggtgagagtgttgg-3′ and 5′-cggacacaagaagaatgcaa-3′; *GA20ox2*, 5′-cggcagattctccactaagc-3′ and 5′-tcgcgctctctctattcaca-3′; *GA20ox3*, 5′-cttaatcagcactcgcacca-3′ and 5′-ccggaatattgaatcgctgt-3′; *GA3ox1*, 5′-ccgaaggtttcaccatcact-3′ and 5′-gaccccaaaggaatgctaca-3′; *GA3ox2*, 5′-tagatcgcatcccattcaca-3′ and 5′-tgaacctaatgcgaaccaca-3′; *GA2ox1*, 5′-ccgaggaacacacttagcaag-3′ and 5′-ggcttcaacaattcgaaagg-3′; *GA2ox2*, 5′-gagtgactcgtgcctgagac-3′ and 5′-ccttgtatgagagtagtcat-3′; *GA2ox3*, 5′-tggtagaggaagagctaaag-3′ and 5′-ctaagcttggtgactatagg-3′; *RGA*, 5′-agaagcaatccagcaga-3′ and 5′-gtgtactctcttcttaccttc-3′; *SLY1*, 5′-gcgcagtactaccgactctg-3′ and 5′-cgagaagatgagtttcactaag-3′; *AtGID1a* (At3g05120), 5′-actctttgtcgcaggcttgt-3′ and 5′-tcgggctaaacggattacac-3′; *AtGID1b* (At3g63010), 5′-taccaacctgcgtctctcct-3′ and 5′-ttccccaacactttgactcc-3′; *AtGID1c* (At5g27320), 5′-accgtcatctcgcagagttt-3′ and 5′-tccttgactcaaccgctctt-3′; *F3′H*, 5′-ggacaccgatggagactgtt-3′ and 5′-cagtccaccgtacttgctga-3′; *LDOX*, 5′-ctaacaacgcgagtggacaa-3′ and 5′-cggagactcaacactcacca-3′; *UF3GT*, 5′-gtgttctgcgctttcggtag-3′ and 5′-aaaaccagagtcagtcaaaacaca-3′; *AtPAP1*, 5′-tgtcccccttttctgttgtc-3′ and 5′-attcctacaacaccggcact-3′; *18S RNA*, 5′-atacgtgcaacaaaccc-3′ and 5′-ctacctccccgtgtca-3′; *TUB*, 5′-tttggagcctgggactatggat-3′ and 5′-acgggggaatgggatgagat-3′.

GA Analysis

Wild-type seeds were surface sterilized and placed on GM medium plates at 4°C for 4 d to synchronize germination. Plates were placed in vertical orientation in controlled-environment chambers (22°C, 16-h light). Four-day-old seedlings were transferred to LP medium for 5 d as described above. Then, seedlings were collected and homogenized in 80% methanol. GA was extracted and separated using HPLC and GA was analyzed using gas chromatography-mass spectrometry methods as described (Eriksson et al., 2006).

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