

# Analysis of Phosphate Acquisition Efficiency in Different Arabidopsis Accessions

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The morphological and physiological characteristics of Arabidopsis accessions differing in their phosphate acquisition efficiencies (PAEs) when grown on a sparingly soluble phosphate source (hydroxylapatite) were analyzed. A set of 36 accessions was subjected to an initial PAE evaluation following cultivation on synthetic, agarose-solidified media containing potassium phosphate (soluble) or hydroxylapatite (sparingly soluble). From the five most divergent accessions identified in this way, *C24*, *Co*, and *Cal* exhibited high PAEs, whereas *Col-0* and *Te* exhibited low PAEs. These five accessions were analyzed in detail. Significant differences were found in root morphology, phosphate uptake kinetics, organic acid release, rhizosphere acidification, and the ability of roots to penetrate substrates. Long root hairs at high densities, high uptake per unit root length, and high substrate penetration ability in the efficient accessions *C24* and *Co* mediate their high PAEs. The third accession with high PAE, *Cal*, exhibits a high shoot-to-root ratio, long roots with long root hairs, and rhizosphere acidification. These results are consistent with previous observations and highlight the suitability of using Arabidopsis accessions to identify and isolate genes determining the PAE in plants.

Phosphorus is a major mineral nutrient required by plants, but is one of the most immobile, inaccessible, and unavailable nutrients present in soils (Holford, 1997). In most soils, phosphorus is available primarily as cation precipitates or poorly soluble organic compounds or is bound to soil particles. The main inorganic phosphates in the soil are iron, aluminum, and calcium phosphates. Iron and aluminum phosphates are the major phosphate compounds in acidic soils, whereas calcium phosphates dominate in neutral to alkaline soil. At higher pH, calcium phosphate is present in the soil as hydroxylapatite [ $\text{Ca}_5(\text{PO}_4)_3\text{OH}$ ] or substituted apatite. Because these inorganic forms of phosphorus are sparingly soluble and organic phosphate must be enzymatically cleaved before uptake, phosphorus is often not readily accessible to plants. Its availability is a major growth-limiting factor for plants in many soils (Barber et al., 1963). The amelioration of phosphate deficiency by the application of costly and environmentally hazardous phosphate fertilizers is not an ideal solution and has raised serious concerns about the continued viability of contemporary agriculture practice. This has led to a search for more environmentally friendly and economically feasible strategies to improve crop production in low phosphorus soils. In an ideal manner, such strategies should enable the efficient use of phosphorus already present in the soil. A prerequisite for strategy development is the identification of

plant traits that limit or enhance the uptake and utilization of phosphorus.

Plant species differ in the efficiency with which they acquire and utilize phosphorus. Phosphate acquisition efficiency (PAE) relates to the different extents to which plants are able to mobilize phosphorus from poorly soluble sources or to take up the soluble phosphorus available in the soil solution. Higher plants show several morphological and physiological adaptations that enable them to acquire phosphorus from sparingly soluble phosphorus soil fractions. These adaptations include root system enlargement (Anghinoni and Barber, 1980; Lynch, 1995), arbuscular mycorrhiza establishment (Smith et al., 1993), increased organic acid exudation (Johnson et al., 1996), rhizosphere acidification (Moorby et al., 1988), increased production of phosphatases (Duff et al., 1989; Goldstein, 1992; Löffler et al., 1992; Green, 1994; Bariola et al., 1994), and enhanced phosphate uptake rate (Schachtman et al., 1998). The genetic characterization of these mechanisms in crop plants is difficult because of the high cost and the large amount of time required for analysis. Furthermore, the analysis of these mechanisms is complicated by the existence of several potential sites of control and by the presence of adaptive responses. Screening programs directed toward the identification of adaptations in efficient genotypes should target quantitative processes and take into account the environmental factors that modify them (Smith et al., 1993).

The genetic model plant Arabidopsis, with its worldwide distribution, range of observed morphological variation, and its ability to adapt to different habitats and photoperiods (Karlsson et al., 1993) provides a good system with which to analyze phos-

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phate efficiency. With the exception of mycorrhizal infections, all of the major characteristic responses of crop plants to phosphate starvation (such as changes in root-to-shoot ratio, anthocyanin accumulation in shoots, and increased phosphatase activity) also occur in Arabidopsis (Trull et al., 1997). In addition, Arabidopsis, due to its small size, can be grown at a high density under defined nutrient conditions, which facilitates the analysis of PAE. An approximate 400 diverse Arabidopsis accessions collected from different geographical locations are available through standardized sources including the following Arabidopsis stock centers: Arabidopsis Biological Resource Center, SENDAI Arabidopsis Seed Stock Center, and Nottingham Arabidopsis Stock Centre. The natural variations in PAE among these accessions provide a valuable resource for analyzing phosphate efficiency. One goal of this study was to characterize the variation in phosphorus acquisition from hydroxylapatite (HA), one of the major forms of phosphorus in alkaline soils, among Arabidopsis accessions. The other goal was to reveal potential morphological and physiological mechanisms involved in achieving high PAEs.

## RESULTS

### HA-PAE of Five Divergent Arabidopsis Accessions

A set of 36 different Arabidopsis accessions was subjected to a pre-screen for differences in phosphorus accumulation on HA medium. The five most divergent accessions selected for this study were C24, Co, and Cal with the highest and Col-0 and Te with the lowest abilities of HA-PAE. These accessions were studied in detail for PAE-related characteristics.

The five selected Arabidopsis accessions were evaluated in detail for their PAEs. To this end, shoot dry masses (Table I) and shoot phosphate contents (Table II) were measured for plants grown in modified MS medium (0.15% or 0.4% [w/v] agarose) containing HA (0.5 g L<sup>-1</sup>) or soluble phosphate (1 mM KH<sub>2</sub>PO<sub>4</sub>), respectively. The shoot dry mass values for accessions C24, Co, and Cal grown in HA medium solidi-

fied with 0.4% (w/v) agarose were higher than those of Col-0 and Te. When calculated as a percentage of those accumulated on KH<sub>2</sub>PO<sub>4</sub> medium, the shoot dry masses of C24, Co, and Cal accessions ranged between 30% and 31%, whereas those of Col-0 and Te were significantly lower and ranged between 21% and 25%. In accordance with this, the latter two accessions were classified as inefficient.

This classification was confirmed by the analysis of the shoot phosphate contents accumulated when the plants were grown under phosphate-limiting conditions (in hydroxylapatite medium solidified with 0.4% [w/v] agarose). C24, Co, and Cal accumulated significantly more phosphate than Col-0 and Te (Table II). Although C24 and Co acquired more phosphate when supplied with HA, they showed relatively low phosphate contents after growth in soluble phosphate medium (phosphate-sufficient conditions). In comparison to the other accessions, Cal attained relatively high phosphate contents and Te relatively low phosphate contents regardless of the phosphate sources provided. C24 and Co showed the highest efficiencies in phosphorus acquisition from HA (expressed as a percentage of phosphorus accumulated from HA/phosphorus accumulated from KH<sub>2</sub>PO<sub>4</sub>), which were approximately 2.5 times greater than those of the less efficient accessions Col-0 and Te (Table II). The PAE of the accession Cal was intermediate between the two groups.

To study the effect of growth medium agar density on phosphate efficiency, the five Arabidopsis accessions were grown on low-density (0.15% [w/v] agarose) modified MS medium containing HA (0.5 g L<sup>-1</sup>) or soluble phosphate (1 mM KH<sub>2</sub>PO<sub>4</sub>). The shoot dry masses of efficient accessions C24 and Co grown in HA calculated as a percentage of those in KH<sub>2</sub>PO<sub>4</sub> medium under these conditions were, however, lower than those of Cal, Col-0, and Te (Table I).

All accessions accumulated more phosphate when grown in low-density (0.15%) agarose HA medium as compared with their phosphate contents on high-density agarose (0.4%) HA medium. The relative differences in phosphorus accumulation from the two

**Table I.** Shoot dry masses of five Arabidopsis accessions after growth in media containing HA or KH<sub>2</sub>PO<sub>4</sub>, respectively.

For each measurement, accession seedlings were grown for 24 d in modified Murashige and Skoog (MS) media (pH 7.0) containing sparingly soluble phosphate (0.5 g L<sup>-1</sup> HA) or soluble phosphate (1 mM KH<sub>2</sub>PO<sub>4</sub>) solidified with 0.15% (w/v) agarose or 0.4% (w/v) agarose. Data are means of three replicate measurements of 10 pooled shoots each. Significant differences within columns ( $P \leq 0.05$ ) are indicated by different characters.

Accession	Shoot Dry Mass on MS Medium Solidified with 0.15% (w/v) Agarose		Shoot Dry Mass on MS Medium Solidified with 0.4% (w/v) Agarose		Shoot Dry Mass on HA as a Percentage of Those on KH <sub>2</sub> PO <sub>4</sub>	
	HA (0.5 g L <sup>-1</sup> )	KH <sub>2</sub> PO <sub>4</sub> (1 mM)	HA (0.5 g L <sup>-1</sup> )	KH <sub>2</sub> PO <sub>4</sub> (1 mM)	0.15% Agarose	0.4% Agarose
	<i>mg shoot<sup>-1</sup></i>					
C24	1.43 c	5.52 b	1.64 a	5.23 b	26 d	31 a
Co	1.48 c	5.58 b	1.71 a	5.57 b	27 d	31 a
Cal	1.87 b	6.2 ab	1.75 a	5.83 ab	30 c	30 a
Col-0	2.64 a	7.1 a	1.28 b	6.00 a	37 b	21 b
Te	1.6 c	3.9 c	0.97 c	3.87 c	40 a	25 b

**Table II.** Phosphate acquisition and PAE of five *Arabidopsis* accessions

For each measurement, seedlings were grown for 24 d in modified MS media (pH 7.0) containing sparingly soluble phosphate (0.5 g L<sup>-1</sup> HA) or soluble phosphate (1 mM KH<sub>2</sub>PO<sub>4</sub>) solidified with 0.4% or 0.15% (w/v) agarose. Data are means of three replicate measurements of 10 pooled shoots each. Significant differences between accessions ( $P \leq 0.05$ ) are indicated by different characters. Mean PAE (%) = mean (phosphate contents in shoot [nmol shoot<sup>-1</sup>] under phosphate-deficient conditions [0.5 g L<sup>-1</sup> HA]/phosphate contents [nmol shoot<sup>-1</sup>] under normal phosphate supply [1 mM KH<sub>2</sub>PO<sub>4</sub>]) × 100.

Accession	Plants Grown in Modified MS Medium Solidified with 0.15% (w/v) Agarose (P contents in shoot)					Plants Grown in Modified MS Medium Solidified with 0.4% (w/v) Agarose (P contents in shoot)				
	0.5 g L <sup>-1</sup> HA		1 mM KH <sub>2</sub> PO <sub>4</sub>		PAE	0.5 g L <sup>-1</sup> HA		1 mM KH <sub>2</sub> PO <sub>4</sub>		PAE
	nmol mg <sup>-1</sup>	Dry wt shoot nmol shoot <sup>-1</sup>	nmol mg <sup>-1</sup>	Dry wt shoot nmol shoot <sup>-1</sup>		nmol mg <sup>-1</sup>	Dry wt shoot nmol shoot <sup>-1</sup>	nmol mg <sup>-1</sup>	Dry wt shoot nmol shoot <sup>-1</sup>	
C24	90 a	130 cd	213 b	1,173 b	11.0 ab	61 a	100.5 a	236 b	1,242 d	8.1 a
Co	98 a	136 c	217 b	1,206 b	11.8 a	60 a	102.5 a	253 b	1,404 c	7.3 a
Cal	85 a	159 b	326 a	2,025 a	7.9 c	61 a	106.0 a	406 a	2,370 a	4.5 b
Col-0	77 a	203 a	304 a	2,170 a	9.3 bc	57 a	73.0 b	358 a	2,150 b	3.4 c
Te	82 a	115 d	292 a	1,154 b	11.4 ab	47 b	45.5 c	366 a	1,410 c	3.2 c

media were more dramatic in the inefficient accessions, *Col-0* and *Te*, than in the efficient accessions, *C24* and *Co*. While the latter accumulated 37% and 54% more phosphate from the 0.15% (w/v) agarose medium, the increases in *Col-0* and *Te* were 213% and 211%, respectively. The relative differences in phosphate concentration in the shoot tissue (nmol mg<sup>-1</sup>) of the plants grown on the two media were, however, unchanged (Table II). When grown in the low-density medium, the less efficient accession *Col-0* acquired 203 nmol phosphate shoot<sup>-1</sup> from sparingly soluble sources, which is approximately 50% more than that of efficient accessions *C24* and *Co*. Furthermore, the differences in PAE between the accessions were less pronounced under these conditions and the ranking of the accessions was different.

#### Characterization of Morphological and Physiological Attributes Related to PAE among the Five Divergent *Arabidopsis* Accessions

Previous studies on plant responses to phosphate deficiency have shown that plant characteristics likely to be involved in the determination of the PAE include seed size (Sadeghian, 1991; Liao and Yan, 1999), root system size (Lynch and van Beem, 1993; Gahoonia et al., 1997), phosphate uptake rates (Schachtman et al., 1998), proton release (Moorby et al., 1988), and organic acid excretion (Van den Boogaard et al., 1992). In this study experiments were

carried out to evaluate these attributes and to determine their relative contributions to the PAEs of the five divergent *Arabidopsis* accessions.

#### Seed Size

The comparison of seed masses (mg<sup>-1</sup> 1,000 seeds) of the five accessions shows that the efficient accession *Cal* has slightly higher seed mass. The other four accessions did not differ significantly in their seed masses (Table III). Therefore, variation in seed size could not account for the difference in dry masses observed for the accessions grown in hydroxylapatite medium.

#### Shoot-to-Root Ratios

It has been generally observed that relative root growth appears to decrease with increasing phosphorus levels, whereas an adequate supply of inorganic phosphate significantly increases the formation of shoot dry matter. To determine whether the differences in PAE among the five *Arabidopsis* accessions are due to unequal growth partitioning, the shoot-to-root dry mass ratios of the accessions were compared. The ranking of shoot-to-root dry mass ratios of the five *Arabidopsis* accessions was found to be: *Cal* > *C24* ≈ *Co* > *Col-0* ≈ *Te*. The accession *Cal* had the highest shoot-to-root ratios in all measure-

**Table III.** Seed sizes and shoot-to-root ratios of five *Arabidopsis* accessions

One thousand dried seeds were sampled and weighed. Each value is the mean ± SD of three determinations. For each shoot-to-root ratio measurement, 25 seedlings were grown for 24 d in modified MS media (pH 7.0) containing sparingly soluble phosphate (0.5 g L<sup>-1</sup> HA) or soluble phosphate (1 mM KH<sub>2</sub>PO<sub>4</sub>) solidified with 0.15% (w/v) agarose. Data are means ± SD of three replicate measurements of 10 pooled shoots and roots. Significant differences within *Arabidopsis* accessions ( $P \leq 0.05$ ) are indicated by different characters.

Accessions	<i>C24</i>	<i>Co</i>	<i>Cal</i>	<i>Col-0</i>	<i>Te</i>
1,000 Seeds dry mass (mg)	17.3 ± 0.4 b	17.0 ± 0.4 b	18.2 ± 0.6 a	16.5 ± 0.4 c	16.7 ± 0.3 bc
Shoot/root at 0.5 g L <sup>-1</sup> HA	1.66 ± 0.21 b	1.63 ± 0.16 b	2.59 ± 0.10 a	1.51 ± 0.11 c	1.54 ± 0.13 c
Shoot/root at 1 mM KH <sub>2</sub> PO <sub>4</sub>	3.12 ± 0.30 bc	3.44 ± 0.49 b	4.05 ± 0.31 a	2.70 ± 0.82 c	2.07 ± 0.55 c

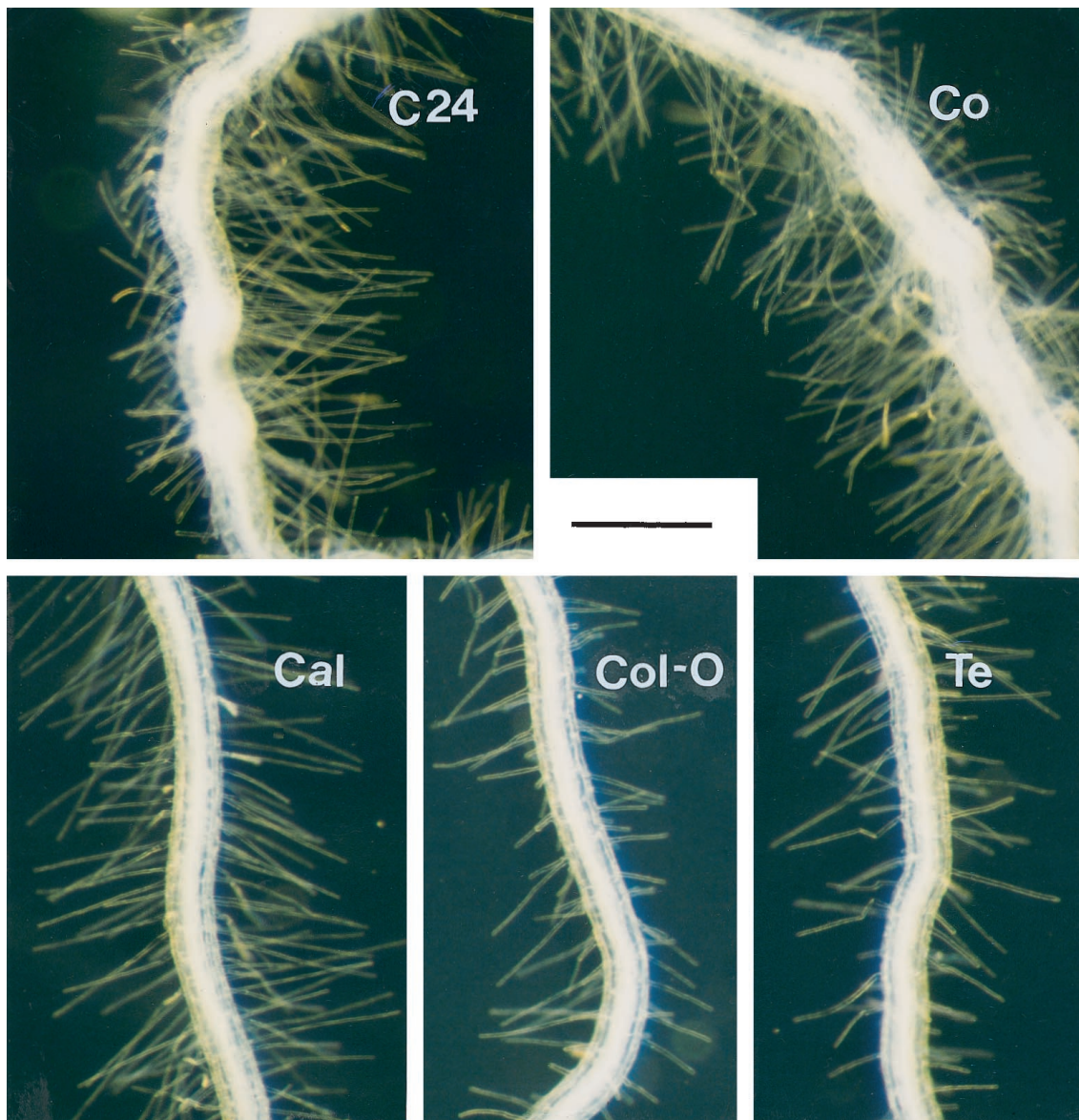
ments. When grown in HA medium all accessions showed a decreased shoot-to-root ratio in comparison to the situation in the soluble phosphate medium. The decrease in the efficient accessions (*C24*, *Co*, and *Cal*), however, was lower in comparison to that of the inefficient accessions (*Col-0* and *Te*). In general, the efficient accessions appeared to have higher shoot-to-root ratios than the inefficient accessions (Table III).

### Root Morphology

A number of root traits, including the size and structure of the root system, appear to affect PAE (Bates and Lynch, 1996). Therefore, it was desirable

to determine the morphological differences in the root system among selected accessions to provide basic architectural information and to detect its potential correlations with the observed differences in PAE. The accessions differed significantly in root length, root hair length, and root hair density (Fig. 1; Tables IV and V).

When grown in 0.4% (w/v) agarose medium, the ranking of accessions according to their root length was *Cal* > *Col-0* > *C24* > *Co* > *Te*. The efficient accessions *C24* and *Co* had relatively short roots, but their root hairs were the longest and had the highest density (Tables IV and V). Root hair length and density was intermediate in *Cal* and low for the less efficient accessions, *Col-0* and *Te*. The high phosphate



**Figure 1.** Root hairs in the primary roots of five Arabidopsis accessions. Seedlings were germinated and grown for 16 d in phosphate-free medium solidified with 0.15% (w/v) agarose. The regions of the roots where root hairs ceased to increase and had a uniform maximum size were observed under a binocular microscope and photographed. Bar represents 0.5 mm.

**Table IV.** Root lengths, root diameters, and substrate penetration abilities of five *Arabidopsis* accessions

Root parameters were determined by microscopic observation of 16-d-old-seedlings grown in phosphate-free MS medium solidified with 0.4% (w/v) or 0.15% (w/v) agarose. Data are means  $\pm$  SD of three replicate analyses of 20 seedling roots each. Significant differences within columns ( $P \leq 0.05$ ) are indicated by different characters. Mean penetration ability (%) = mean (root length at 0.4% [w/v] agarose MS medium/root length at 0.15% [w/v] agarose MS medium)  $\times$  100.

Accessions	Root Length in MS Medium		Root Diameter in MS Medium		Penetration Ability
	Solidified with 0.4% (w/v) agarose	Solidified with 0.15% (w/v) agarose	Solidified with 0.4% (w/v) agarose	Solidified with 0.15% (w/v) agarose	
	cm		mm		%
<i>C24</i>	1.30 $\pm$ 0.19 c	1.58 $\pm$ 0.30 c	0.18 $\pm$ 0.01	0.18 $\pm$ 0.01	85.0 $\pm$ 18.2 a
<i>Co</i>	1.18 $\pm$ 0.17 d	1.50 $\pm$ 0.28 c	0.18 $\pm$ 0.01	0.18 $\pm$ 0.01	79.6 $\pm$ 19.6 a
<i>Cal</i>	1.85 $\pm$ 0.40 a	2.80 $\pm$ 0.45 a	0.17 $\pm$ 0.01	0.17 $\pm$ 0.01	74.0 $\pm$ 25.6 a
<i>Col-0</i>	1.55 $\pm$ 0.19 b	3.02 $\pm$ 0.44 a	0.16 $\pm$ 0.01	0.16 $\pm$ 0.01	53.4 $\pm$ 11.8 b
<i>Te</i>	1.00 $\pm$ 0.23 e	2.34 $\pm$ 0.60 b	0.16 $\pm$ 0.01	0.16 $\pm$ 0.01	43.7 $\pm$ 12.7 b

efficiency observed in *C24* and *Co* thus coincided with the presence of long, dense root hairs.

When grown in 0.15% (w/v) agarose medium, plants of all accessions developed longer roots, but the relative differences in root length were accession specific (Table IV). Thus *Te* displayed the largest difference with 2.3-fold longer roots established in the 0.15% (w/v) agarose medium as compared with the roots grown in 0.4% (w/v) agarose medium. In the 0.15% (w/v) agarose medium *Col-0* had the longest roots, approximately 2 times longer than those of *C24* and *Co*. In accordance with this, the ranking of accessions with respect to their root lengths was *Col-0*  $\approx$  *Cal* > *Te* > *C24*  $\approx$  *Co*. When grown in 0.15% (w/v) agarose medium only *Cal* plants developed considerably longer root hairs as compared with the 0.4% (w/v) agarose medium (Table V). Only very small differences were observed in the root hair densities of plants grown in high and low strength medium within the accessions (Table V). The increased root lengths (and root hair length in *Cal*) in 0.15% (w/v) agarose medium coincided with strong increases in the PAEs of the accessions *Cal*, *Col-0*, and *Te* (Table II).

The observed differences in root lengths established upon growth in the 0.15% and 0.4% (w/v) agarose media were used to derive a measure for the substrate penetration ability of the roots of the different accessions. Determined as the ratios of the root lengths in 0.4% (w/v) agarose medium to the root

lengths in 0.15% (w/v) agarose medium, substrate penetration abilities were higher for roots of the phosphate efficient accessions *C24*, *Co*, and *Cal* than for the inefficient accessions *Col-0* and *Te* (Table IV).

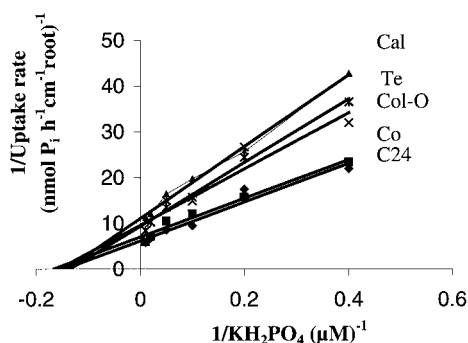
### Phosphate Uptake Kinetics

Accessions may vary in their effectiveness in acquiring phosphate through different levels of expression of transport proteins in roots ( $I_{max}$ ), through differences in the affinity ( $K_m$ ) for phosphate, or through variation in  $C_{min}$  (the minimum phosphate concentration in the growth medium at which no net uptake occurs into the roots) for phosphate. Phosphate uptake of the five *Arabidopsis* accessions was measured using seedlings grown in liquid culture for 7 to 8 d. The results of short-term uptake experiments performed with the plants grown in phosphate-sufficient (0.5 mM  $\text{KH}_2\text{PO}_4$ ) or phosphate-deficient (2.5  $\mu\text{M}$   $\text{KH}_2\text{PO}_4$ ) medium are shown in Table VI. Phosphate uptake over a concentration range of 0 to 300  $\mu\text{M}$  can be described by Michaelis-Menten kinetics (Barber, 1995). Phosphate uptake was measured for concentrations ranging from 2.5 to 100  $\mu\text{M}$   $\text{KH}_2\text{PO}_4$  (Fig. 2). Michaelis-Menten parameters describing the kinetics of phosphorus uptake were estimated separately for each accession by linear regression analysis.

**Table V.** Root hair lengths and densities of five *Arabidopsis* accessions

Root hair parameters were determined by microscopic observation and image analysis of 16-d-old-seedlings grown in phosphate-free modified MS medium solidified with 0.4% (w/v) agarose or 0.15% (w/v) agarose. Data are means  $\pm$  SD of three replicate analyses of 20 seedling roots each. Significant differences within columns ( $P \leq 0.05$ ) are indicated by different characters.

Accessions	Root Hair Length in -P MS Medium		Root Hair Density in -P MS Medium	
	Solidified with 0.4% (w/v) agarose	Solidified with 0.15% (w/v) agarose	Solidified with 0.4% (w/v) agarose	Solidified with 0.15% (w/v) agarose
	mm		number $\text{mm}^{-1}$ rootlength	
<i>C24</i>	0.46 $\pm$ 0.09 a	0.51 $\pm$ 0.08 a	66 $\pm$ 9 a	70 $\pm$ 9 a
<i>Co</i>	0.47 $\pm$ 0.08 a	0.50 $\pm$ 0.09 a	64 $\pm$ 10 a	72 $\pm$ 10 a
<i>Cal</i>	0.35 $\pm$ 0.07 b	0.58 $\pm$ 0.08 a	42 $\pm$ 6 b	39 $\pm$ 6 b
<i>Col-0</i>	0.30 $\pm$ 0.07 c	0.35 $\pm$ 0.07 b	38 $\pm$ 7 b	40 $\pm$ 7 b
<i>Te</i>	0.32 $\pm$ 0.13 c	0.39 $\pm$ 0.12 b	39 $\pm$ 6 b	44 $\pm$ 6 b



**Figure 2.** Lineweaver-Burke plots of phosphate uptake into roots of five Arabidopsis accessions. For each measurement, 300 seedlings (on three nylon meshes) were grown in a modified 0.5× Hoagland medium containing 2.5 μM KH<sub>2</sub>PO<sub>4</sub>. Phosphate concentrations in the uptake solutions ranged from 2.5 to 100 μM. Plotted values are means of three replicate measurements.  $Y_{C24} = 42.344X_{C24} + 6.1877$ ,  $R^2_{C24} = 0.9481$ ;  $Y_{Co} = 42.162X_{Co} + 7.0285$ ,  $R^2_{Co} = 0.9723$ ;  $Y_{Cal} = 78.462X_{Cal} + 11.086$ ,  $R^2_{Cal} = 0.993$ ;  $Y_{Col-0} = 61.345X_{Col-0} + 9.6375$ ,  $R^2_{Col-0} = 0.9262$ ;  $Y_{Te} = 69.503X_{Te} + 9.3792$ ,  $R^2_{Te} = 0.9829$ .

The apparent  $K_m$  of the phosphate transporters of all five accessions were determined to be approximately 6.0 to 7.0 ( $\pm 2.0$ ) μM, which is characteristic for high affinity transporters. Although there were no significant differences among accessions for the estimated apparent  $K_m$  value for phosphate-starved plants, the estimated  $I_{max}$  (pmol h<sup>-1</sup> cm<sup>-1</sup> root) values for C24 and Co were greater by a factor of about 1.5 than those of Cal, Co, and Col-0. Thus phosphate-starved C24 and Co plants show the highest phosphate uptake rates per unit root length. On a plant biomass basis, however, the uptake rates of Cal, Col-0, and Te are higher (Table VI). This difference is due to the variation in root lengths, which, under the condition used, were greater for Cal, Col-0, and Te than for C24 and Co.

Another physiological parameter  $C_{min}$  that can affect nutrient absorption was determined for the five accessions, but no obvious differences were ob-

served. All accessions were able to deplete the 0.5-μM KH<sub>2</sub>PO<sub>4</sub> solutions to between 7 and 11 nM (Table VI).

### Organic Acid Exudations

Organic acids released from the roots of phosphorus-efficient plants increase the availability of phosphorus by mobilizing sparingly soluble forms of phosphate such as calcium-phosphate (Dinkelaker et al., 1989). To determine if a similar mechanism operates in Arabidopsis, the accessions were examined for organic acid root exudation triggered by phosphate deficiency. Citric, malic, and succinic acids were measured in the root exudates of 8-d-old plants. The amount of organic acids released from roots of phosphorus-starved plants (grown at 10 μM KH<sub>2</sub>PO<sub>4</sub>) differed from those of plants grown in phosphorus-sufficient conditions (0.5 mM KH<sub>2</sub>PO<sub>4</sub>). When plants were supplied with adequate phosphorus, organic acids were released in very low amounts by all accessions. In contrast, these acids were present in higher concentrations in the bathing solutions when plants were pre-cultivated for 8 d under phosphate deficient conditions. The amounts of citric and malic acids released from the roots differed among accessions (Table VII; Fig. 3).

In all accessions citrate and malate are the predominant organic acids released. The kinds and amounts of exuded organic acids appeared to be accession specific. In the accessions C24, Co, and Col-0, citrate exudation is slightly higher than is the exudation of malate, whereas in the accessions Cal and Te, malate is released in higher amounts than is citrate (in Te, malate exudation is six times higher than citrate).

In the efficient accessions C24 and Co the amounts of citrate and malate (μmol h<sup>-1</sup> g<sup>-1</sup> dry mass plants) in root exudates are higher than those of Cal, Col-0, and Te. When calculated on the basis of root lengths, these differences are even more dramatic. The accumulation of organic acids in the rhizosphere of C24 and Co plants is therefore expected to be drastically higher than that of Cal, Col-0, and Te plants (Table VII).

**Table VI.** Phosphate uptake kinetic parameters of five Arabidopsis accessions

The apparent kinetic parameters  $K_m$  and  $I_{max}$  were determined by plotting phosphate uptake for 1 h as a function of phosphate concentrations. Uptake experiments were performed with 7- to 8-d-old-seedlings pre-grown in modified 0.5× Hoagland medium containing 500 or 2.5 μM KH<sub>2</sub>PO<sub>4</sub>, respectively.  $C_{min}$ s were determined through 7 h depletion of a solution initially containing 0.5 μM KH<sub>2</sub>PO<sub>4</sub> by 10-d-old-seedlings pre-grown in modified 0.5× Hoagland medium lacking phosphate. Data are means  $\pm$  SD of three replicate measurements of 300 seedlings each. Significant differences within columns ( $P \leq 0.05$ ) are indicated by different characters.

Accessions	Apparent $K_m$		$I_{max}$				$C_{min}$
	500 μM Phosphate	2.5 μM Phosphate	500 μM Phosphate	2.5 μM Phosphate	500 μM Phosphate	2.5 μM Phosphate	
	μM		pmol h <sup>-1</sup> cm <sup>-1</sup> rootlength		nmol h <sup>-1</sup> g <sup>-1</sup> fresh wt plant		
C24	3.64 $\pm$ 0.72	6.84 $\pm$ 1.10	103 $\pm$ 19 a	162 $\pm$ 35 a	118 $\pm$ 22 b	322 $\pm$ 70 b	9 $\pm$ 3 ab
Co	3.44 $\pm$ 0.65	6.00 $\pm$ 1.32	99 $\pm$ 22 a	142 $\pm$ 25 a	117 $\pm$ 26 b	300 $\pm$ 53 b	10 $\pm$ 3 a
Cal	4.08 $\pm$ 1.26	7.07 $\pm$ 1.45	85 $\pm$ 18 ab	90 $\pm$ 22 b	181 $\pm$ 38 a	447 $\pm$ 69 a	11 $\pm$ 4 a
Col-0	3.92 $\pm$ 1.32	6.37 $\pm$ 1.61	74 $\pm$ 11 ab	104 $\pm$ 10 b	150 $\pm$ 22 a	440 $\pm$ 42 a	7 $\pm$ 1 b
Te	3.91 $\pm$ 1.09	7.40 $\pm$ 2.1	62 $\pm$ 15 b	107 $\pm$ 16 b	149 $\pm$ 36 a	494 $\pm$ 74 a	11 $\pm$ 3 a

**Table VII.** Organic acid exudates from roots of five *Arabidopsis* accessions

For each measurement, 400 seedlings (on four nylon meshes each with 100 seedlings) were grown for 8 d in a modified 0.5× Hoagland nutrient solution containing 10  $\mu\text{M}$   $\text{KH}_2\text{PO}_4$  or 0.5 mM  $\text{KH}_2\text{PO}_4$ , respectively. Root exudates were collected in water for 1 h, concentrated, and analyzed using HPLC. Data are means  $\pm$  SD of three replicate measurements with 400 seedlings each. Significant differences within columns ( $P \leq 0.05$ ) are indicated by different characters.

Accessions	Organic Acids Exudation				Organic Acids Exudation			
	0.5 mM Phosphate		10 $\mu\text{M}$ Phosphate		0.5 mM Phosphate		10 $\mu\text{M}$ Phosphate	
	Citrate	Malate	Citrate	Malate	Citrate	Malate	Citrate	Malate
	$\mu\text{mol h}^{-1} \text{g}^{-1} \text{dry wt plant}$				$\text{pmol h}^{-1} \text{cm}^{-1} \text{root}$			
<i>C24</i>	2.6 $\pm$ 1.8 a	2.2 $\pm$ 2.0 a	12.2 $\pm$ 4.9 a	10.0 $\pm$ 4.9 a	172 $\pm$ 125 a	145 $\pm$ 132 a	428 $\pm$ 172 a	391 $\pm$ 172 a
<i>Co</i>	2.0 $\pm$ 1.5 ab	2.0 $\pm$ 1.8 a	13.6 $\pm$ 3.4 a	11.6 $\pm$ 4.9 a	120 $\pm$ 90 ab	120 $\pm$ 108 a	440 $\pm$ 125 a	426 $\pm$ 180 a
<i>Cal</i>	0.9 $\pm$ 0.8 b	1.6 $\pm$ 1.0 ab	4.5 $\pm$ 1.8 b	8.5 $\pm$ 2.2 a	33 $\pm$ 29 b	59 $\pm$ 36 ab	90 $\pm$ 36 b	169 $\pm$ 43 b
<i>Col-0</i>	0.5 $\pm$ 0.5 b	0.5 $\pm$ 0.2 b	2.8 $\pm$ 1.0 c	2.0 $\pm$ 1.0 b	18 $\pm$ 18 b	18 $\pm$ 8 c	60 $\pm$ 21 bc	42 $\pm$ 21 c
<i>Te</i>	0.6 $\pm$ 0.5 b	2.0 $\pm$ 1.0 a	1.9 $\pm$ 1.0 c	11.9 $\pm$ 5.9 a	14 $\pm$ 12 b	48 $\pm$ 24 b	29 $\pm$ 16 c	186 $\pm$ 92 b

### pH Changes and Its Effect on Phosphate Solubility

In alkaline soils, phosphorus mobilization occurs predominantly through rhizosphere acidification. To test whether organic anions are released in association with protons and whether rhizosphere acidification is correlated with organic anion secretion in *Arabidopsis*, pH changes in the growth media were measured.

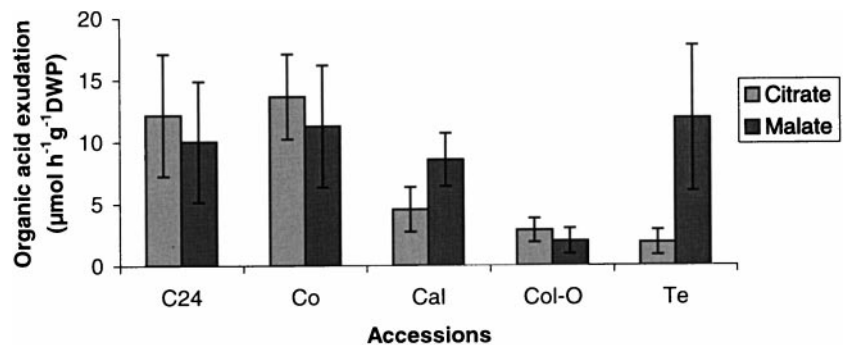
As expected, since nitrogen was supplied exclusively in the  $\text{NO}_3^-$  form, cultivation of plants from all accessions resulted in pH increase of the medium. The observed pH changes in the growth medium, however, did not correlate to organic acid exudation. No significant differences in the pH of growth medium of high organic acid exuding, phosphate-efficient accessions (*C24* and *Co*) and low organic acid exuding, phosphate-inefficient accessions (*Col-0* and *Te*) were observed. Of all the accessions only *Cal* showed a slightly enhanced release of protons (Table VIII). Consistent with this observation, the accession *Cal* showed the highest PAE at higher buffer concentration (100 mM MOPS [3-(*N*-morpholino)propanesulfonic acid], pH 7.0; data not shown).

### DISCUSSION

By analysis of *Arabidopsis* accessions, morphological and physiological traits that are likely to affect the PAE of higher plants were identified. At first the

experiments were focused on identifying *Arabidopsis* accessions exhibiting PAE variations. Five divergent accessions selected after screening 36 accessions were analyzed in detail to characterize the adaptations responsible for PAE on HA. Different results may be obtained upon supply of the plants with other forms of sparingly soluble phosphate such as organic phosphate, iron phosphate, and aluminum phosphate. To be able to modify precisely and reproducibly growth conditions such as phosphorus source and supply, as well as the strength of the substrate, the experiments were performed in agarose-solidified modified MS medium. This experimental setup furthermore allowed in situ root morphology analysis. Similar conditions were used in previous studies on phosphate uptake in *Arabidopsis* accessions (Krannitz et al., 1991), on isolation and characterization of the *Arabidopsis* *pho1* and *pho2* mutants (Poirier et al., 1991; Delhaize and Randall, 1995), as well as mutants altered in organic acid exudation (Larsen et al., 1996). Potential effects of oxygen gradients in the media (Chung and Ferl, 1999) were restricted by the use of a maximum thickness of the media of 4 mm. A potential influence of oxygen gradients on root growth was furthermore excluded by the use of media with different agarose concentrations in which root growth rates were different and in which different relative PAEs were observed. The *C24*, *Co*, and *Cal* accessions showed

**Figure 3.** Organic acid exudations (citrate and malate) by the roots of five *Arabidopsis* accessions. For each measurement, 400 seedlings (on four nylon meshes) were grown for 8 d in modified 0.5× Hoagland nutrient solution containing 10  $\mu\text{M}$   $\text{KH}_2\text{PO}_4$ . Root exudates were collected for 1 h, concentrated, and analyzed using HPLC. Each bar represents the total micromoles of organic acid released per gram seedling dry weight. Data are means  $\pm$  SD of three replicate measurements.



**Table VIII.** pH changes in growth media of five Arabidopsis accessions

For each measurement, 300 seedlings were grown aseptically for 10 d in a phosphate-free modified MS nutrient solution. pH of the growth medium was measured using a pH meter. Data are means  $\pm$  SD of three replicate measurements. Significant differences ( $P \leq 0.05$ ) are indicated by different characters.

Accession Medium	<i>C24</i>	<i>Co</i>	<i>Cal</i>	<i>Col-0</i>	<i>Te</i>	Control Medium
pH	5.65 $\pm$ 0.06 a	5.64 $\pm$ 0.07 ab	5.43 $\pm$ 0.06 d	5.55 $\pm$ 0.08 c	5.60 $\pm$ 0.10 b	4.48 $\pm$ 0.05

relatively high shoot phosphate accumulation when grown in medium containing sparingly soluble phosphate and solidified with 0.4% (w/v) agarose, whereas accessions *Col-0* and *Te* acquired less phosphate under identical conditions. However, accessions *Cal*, *Col-0*, and *Te* showed increased phosphate contents on low-density agarose medium, apparently due to dramatic increases in root length. Variations among these accessions in PAE-related traits such as root morphology (root length, root hair length, and root hair density), transport activity per unit length, organic acid exudation, and root penetration ability have been identified. Most probably, however, the predominant parameter determining the PAEs of the analyzed Arabidopsis accessions is the root morphology affected by the strength of the growth substrate.

#### Effect of Root Morphology and Penetration Ability on Phosphate Efficiency

Root hairs substantially extend the root surface area available for nutrient uptake from the soil. Theoretical calculations (Nye, 1966), autoradiographs (Lewis and Quirk, 1967), mechanistic models (Föhse et al., 1991), and experimental evidence (Gahoonia and Nielsen, 1998) suggest a relationship between root hair length and density and phosphate uptake from the soil. The Arabidopsis accessions tested for root morphology differed widely in their root hair parameters. The accessions *C24*, *Co*, and *Cal* have long, dense root hairs, whereas *Col-0* and *Te* have shorter and fewer root hairs. The contribution of root hairs to phosphate acquisition depends upon the diffusion of nutrients (Barber and Silberbrush, 1984). Long root hairs may intercept the phosphate diffusing toward the root at some distance. If the root hairs are short and develop in pre-existing zones depleted by the absorption of phosphate by the rhizodermis, they contribute only a little to phosphate acquisition. The presence of long and dense root hairs in *C24*, *Co*, and *Cal* might well explain the superior PAE exhibited by these accessions when grown in HA medium. A wide variation in root hairs of cereal varieties and the correlation of long root hairs to high phosphate uptake has been reported (Gahoonia et al., 1997). Root hair growth appears to be controlled by multiple genetic loci and to be strongly influenced by environmental conditions (Schiefelbein and Somerville C, 1990).

Root parameters were measured under two different conditions since it was found that they changed with changes in growth medium density (Tables IV and V). Root elongation of the five accessions decreased when the growth media agarose concentration increased (0.15%–0.4%). The high acquisition of phosphate from sparingly soluble phosphate medium, as well as from full phosphate medium in *Cal* is probably due to its long roots and long root hairs (i.e. high general uptake efficiency). The comparison of the root lengths of the accessions furthermore showed that the efficient accessions *C24* and *Co* have slightly shorter roots as compared with the less efficient accession *Col-0*. *C24* and *Co*, however, display highest root hair densities and root hair lengths among all accessions tested when grown in 0.4% (w/v) agarose medium. The inefficient accessions *Col-0* and *Te* have much shorter and fewer root hairs. However, when grown in low-density agarose medium (0.15% [w/v] agarose), *Col-0* and *Te* showed strongly increased PAEs. In case of *Col-0* this results in a PAE equivalent to those exhibited by the efficient accessions *C24* and *Co*. The change in phosphate efficiency of accessions *Col-0* and *Te* under low agarose density is obviously due to the dramatic increase in their root lengths, and consequently their root surface areas and, therefore, the volume of the medium, which they can exploit.

The development of a root system capable of anchoring the shoot and obtaining sufficient water and nutrients is essential for the survival of most terrestrial plants (Bengough et al., 1997). In the soil, roots experience a range of penetration resistances depending upon soil type. Since the selected accessions were sampled from different habitats, their adaptations potentially included differences in soil penetration abilities. The differential changes in the root growth of the different accessions with varying densities of the agarose medium reflect differences in their penetration abilities. The root penetrating abilities of *C24* and *Co* were found to be approximately two times higher than those of *Col-0* and *Te*. In accordance with this, the *C24* and *Co* accessions showed higher PAEs when grown in high-density agarose medium (0.4%).

Mucilage exudation and sloughing of root cap cells may be involved in decreasing the frictional resistance in soil penetration by roots. Increases in the exudation of mucilage are found in roots growing in



compressed soil when compared with those in loose soil (Boeuf-Tremblay et al., 1995). Whether similar mechanism influences the root penetration ability in *Arabidopsis* is presently unknown.

### Diversity among Accessions in Phosphate Uptake

The results of uptake measurements show that the accessions differed in their phosphate uptake characteristics. Differences in the phosphate uptake have also been identified among crops plants (Barber, 1995; Horst et al., 1996; Römer and Schenk, 1998). In the uptake experiments described here all *Arabidopsis* accessions showed single-phase Michaelis-Menten kinetics when supplied with phosphate in the range of 2.5 to 100  $\mu\text{M}$ . The apparent  $K_m$  values of phosphate uptake, determined for plants of the accessions C24, *Co*, *Cal*, *Col-0*, and *Te* grown under phosphate deficient conditions, are characteristic for high affinity transporters (Schachtman et al., 1998). Similar results for the high affinity system have been reported in *Arabidopsis* despite differences in experimental procedure and age of plants used (Dunlop et al., 1997; Dong et al., 1999). It is surprising that slightly higher affinities were observed for C24, *Co*, *Cal*, *Col-0*, and *Te* as determined by apparent  $K_m$  values (which could be determined here with lower precision) under phosphate-sufficient conditions.

$I_{max}$  values, under phosphate-deficient conditions, were within the range of 90 to 152  $\text{pmol h}^{-1} \text{cm}^{-2}$  root, which is approximately 1.5-fold higher than the  $I_{max}$  values determined under sufficient phosphate supply. Increases in  $I_{max}$  have also been reported for various species under phosphate deficiency by several researchers (Lee 1982; Drew et al., 1984; Dunlop et al., 1997) and have been attributed to increased expression of Pi transporters in tobacco suspension cells (Shimogawara and Usuda, 1995).

Dunlop et al. (1997), however, suggested the existence of a low-affinity system (two-phase kinetics) in *Arabidopsis* at higher phosphate concentrations. Under phosphate-deficient conditions the efficient accessions C24 and *Co* have approximately 1.5 times higher  $I_{max}$  ( $\text{pmol h}^{-1} \text{cm}^{-1}$  root) than those of *Col-0* and *Te* and, consequently, they can acquire more phosphate per centimeter of root than the latter (less efficient) accessions. Because no obvious differences were observed in the apparent  $K_m$  values among the different accessions, the differences in the phosphorus uptake systems of the accessions are probably manifested in different frequencies of active phosphate transporters (difference in  $I_{max}$ ) rather than in expression of different kinds of phosphate transporter genes. Variation in the numbers of functional ion transporters per unit length of root may be caused by differential rates of synthesis and breakdown of the transport proteins, a process likely to cause changes in the maximum transport capacity of the root (Lee, 1982). The tomato phosphate trans-

porter genes *LePT1* and *LePT2* show predominant expression in the root epidermis and the root hairs (Daram et al., 1998; Liu et al., 1998). The phosphate transport activity may therefore also be influenced by the frequencies and sizes of the root hairs, which differ in the various accessions and are highest in C24 and *Co*. The lower phosphate transport activity per centimeter of root in *Cal*, *Col-0*, and *Te* is over-compensated by the elevated root lengths in these accessions when grown in low strength medium. This results in higher phosphate transport activity per gram of plant fresh weight in *Cal*, *Col-0*, and *Te* under these conditions in comparison to C24 and *Co*. The superior PAEs displayed by C24 and *Co* when grown in high-strength medium, therefore, are most probably conferred by a combination of high root penetration abilities, high root surface areas, and high phosphate transport activities per centimeter of root.

Another physiological parameter,  $C_{min}$ , the concentration of phosphate in the growth medium at which no net uptake or release of ions occurs, is a measure of the ability of a plant to acquire phosphate at low concentrations and of the degree to which the soil solution can be depleted. This threshold may be set by a limited affinity of the carrier sites for the ions or it may be the point at which influx is balanced by the efflux (Marschner, 1988). This parameter of ion uptake is also strongly affected by the plant nutritional status (like  $I_{max}$  and  $K_m$ ). Measurement of phosphate-starved plants showed that all tested accessions (C24 and *Co*, *Cal*, *Col-0*, and *Te*) displayed  $C_{min}$  between 7 and 11 nM. In accordance with this, this measure does not contribute substantially to the differences in phosphate efficiency in *Arabidopsis* as in other plant species (Jungk et al., 1990). The  $C_{min}$  values determined in this study were four to six times lower than those measured by Krannitz et al. (1991) in 25 *Arabidopsis* genotypes. The reason for this discrepancy may reside in the different measurement conditions used.

### Phosphate Mobilization by Root Exudates

In addition to root morphology and uptake activity, the release of organic anions from roots can contribute to PAE through the dissolution of sparingly soluble phosphates (Dinkelaker et al., 1989). It is widely believed that the efficiency of uptake is of minor importance for phosphorus acquisition from soils because availability of Pi to the root surface rather than its uptake is the limiting factor (Barber, 1995). The efficient accessions C24 and *Co* exude four to five times more citrate than the less efficient accessions *Col-0* and *Te*. The increased exudation of organic anions, particularly citrate and malate, in the accession C24 and *Co* may enable them to acquire phosphate from hydroxylapatite. Differences in the exudation of citrate and malate among the members

of the *Brassicaceae*, which correlated with their PAEs, have been reported previously (Van den Boogaard et al., 1992). Consistent with these data, elevated root citrate and malate exudation has been observed in the efficient Arabidopsis accessions *C24* and *Co* in this study.

Polar substances like organic acids can diffuse into the rhizosphere due to the high electrochemical potential gradient existing between the cytoplasm of root cells and the soil solution (Jones et al., 1994). This passive diffusion of citrate, however, is slow ranging from 14.4 to 93.6 pmol h<sup>-1</sup> (cm root length)<sup>-1</sup> for wheat and tomato and 100 to 200 pmol h<sup>-1</sup> (cm root length)<sup>-1</sup> from non-proteoid roots of white lupin as compared with the active citrate exudation from the proteoid roots of phosphorus-deficient white lupin, which is approximately 30- to 70-fold higher (Neumann and Römheld, 1999; Neumann et al., 1999). Citric acid exudation rates in white lupin depend upon the age of proteoids roots and reaches up to 6,700 pmol h<sup>-1</sup> (cm root length)<sup>-1</sup> in mature proteoid roots (Neumann et al., 1999). Exudation of organic acids at a high level in response to phosphorus stress has been documented also for other species such as oilseed rape (Hoffland et al., 1992) and alfalfa (Lipton et al., 1987). The carboxylates that are most effective in mobilizing phosphate from sparingly soluble sources are citrate > oxalate > malate (Bar-Yosef, 1996).

The root exudation of citrate and malate in the efficient accessions *C24* and *Co* is lower as compared with white lupin proteoid roots, but it is comparable with oilseed rape (Hoffland et al., 1989). In contrast to white lupin and oilseed rape, however, the high citrate and malate exudation in the Arabidopsis accessions *C24* and *Co* are not accompanied by strong proton extrusion. All accessions displayed a net alkalization of the growth medium. To monitor potential spatial variation of root-induced pH changes, other experimental procedures need to be applied such as the use of pH indicators and H<sup>+</sup>-selective microelectrodes (Marschner and Römheld, 1983; Plassard et al., 1999). Rhizosphere acidification would be required to cause the dissolution of HA resulting in efficient phosphate mobilization. Lowest pHs of the growth media were observed for the accessions *Cal* and *Col-0*, which showed intermediate or low root organic acid exudation, respectively. Therefore, the main mechanism governing the proton release in Arabidopsis probably is the balancing of cation-anion uptake (Jeong and Lee, 1996; Schötteldreier and Falkengren-Grerup, 1999). Rhizosphere acidification in response to phosphorus starvation in oilseed rape has been attributed to unbalanced cation-anion uptake (Hedley et al., 1982). The independence of the rate of organic acid anion release and the degree of HA utilization by the Arabidopsis accessions is furthermore supported by the observation that the differences in their PAEs are minor when

they are grown in low strength media. The ability to exploit a large substrate volume therefore appears to be of much higher importance than activities related to an enhanced phosphate mobilization from the sparingly soluble HA.

Secretion of organic acids without concomitant release of protons has been observed in phosphorus-efficient plants adapted to acid soils (H. Lambers, personal communication). It remains to be tested whether the Arabidopsis accessions *C24* and *Co* display high PAEs under such conditions.

This study highlights the fact that inherent differences in PAE exist among Arabidopsis accessions. The adaptations evolved in these accessions to cope with low phosphate availability in soils are dominated by root growth and morphology and by phosphate uptake rates per unit root length. Rhizosphere acidification, root organic acid exudation, and affinity of the phosphate transporters appear to be of minor relevance. Two extreme sets of accessions were identified: The first, represented by *C24* and *Co*, develops relatively short roots with high substrate penetration abilities, long root hairs at high densities, and high phosphate uptake efficiencies per unit root length. The second, represented by *Col-0* and *Te*, produces long roots (under favorable conditions) with low substrate penetration abilities, short and sparse root hairs, and low uptake efficiencies per unit root length. *Cal* appears intermediate for these characteristics, but displays slightly enhanced proton extrusion.

Plants with larger root systems have a greater potential to acquire phosphate than do plants with small roots. However, the uptake experiments show that the accessions with small root systems can be more efficient because they can acquire more phosphate per unit length of root. This finding is in close agreement with the conclusions reached through other studies (Evans 1977; Caradus, 1980; Rao et al., 1997). In most soils the phosphorus available to the plant is concentrated primarily in the upper soil horizons and decreases with the soil depth (Anderson, 1980; Keter and Ahn 1986; Pothuluri et al., 1986; Lynch 1995). When this is the case plants with short roots, but long root hairs at high density and with root systems of branched architecture would be more efficient than accessions with long roots and short and sparse root hairs. The accessions *C24* and *Co* with their slow, but sustainable (high penetration ability) root growth and high phosphate uptake per unit of root are apparently better adapted to compact soils, whereas the less efficient accessions with fast growing roots such as *Col-0* and *Te* are better suited to loose soils. The latter cope with phosphate deficiency through elongation of their roots so that they can access a larger soil volume, which may be of lower richness.

Taking into consideration multiple soil parameters, Barber (1995) predicted that nutrient uptake is likely

to be most strongly affected by the root growth rate. This is supported by the observed strong drop in the PAEs of the *Col-0* and *Te* accessions upon increased growth medium density, which caused severe root growth retardation. Under these conditions, the accessions *C24* and *Co*, with their higher root penetration ability and their long root hairs and high uptake per centimeter of root are more efficient.

In conclusion this study represents a detailed morphological and physiological analysis of the mechanisms governing PAEs of Arabidopsis accessions. Based on the results presented here the excellent resources available for this molecular genetic model system can be used to isolate genes that determine phosphate efficiency traits. Furthermore, favorable/unfavorable allelic variants of these genes may be identified that shall provide detailed insight into the molecular mechanisms underlying the PAE phenomenon.

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

Seeds of the Arabidopsis accessions *Ag-0*, *An*, *Bch-1*, *Bla*, *Bur*, *Cal*, *Cen*, *Co*, *Cvi*, *Ei*, *Eil-0*, *En*, *Gr*, *Hi*, *Kil*, *LalIII*, *Lip-0*, *Lm*, *Lu*, *M4*, *No*, *Ob-0*, *Old-1*, *Oy*, *Pa*, *Per*, *Pr*, *Rsch*, *Sue*, *Sg-1*, *Te*, and *Yo* were obtained from Dr. Simon Misera (Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Germany). Further accessions were represented by the lab strains *C24*, *Col-0*, *Ler*, and *Ws*. To identify the Arabidopsis accessions with high and low PAEs, seeds of these 36 accessions from a range of different geographical origins were germinated in modified MS medium (Murashige and Skoog, 1962) solidified with 0.4% (w/v) agarose. Ammonium nitrate was replaced with potassium nitrate to avoid acidification resulting from ammonium uptake. For HA [ $\text{Ca}_5(\text{PO}_4)_3\text{OH}$ ] medium or soluble phosphate medium, HA ( $0.5 \text{ g L}^{-1}$ ) or monopotassium dihydrogen phosphate (1 mM), respectively, was included as the sole phosphate source. Through the addition of 10 mM MOPS, the growth medium was buffered to pH 7.0. HA is sparingly soluble at pH 7.0 and was therefore added as a suspension. The seedlings were germinated and cultivated at 22°C and a 16-h light period at  $60$  to  $90 \mu\text{mol m}^{-2} \text{ s}^{-1}$ . After 24 d of culture, 10 shoots of each accession were pooled, blotted between tissue paper, dried overnight at 60°C, weighed, and their phosphate contents were determined according to Ames (1966). On the basis of these data the five most divergent accessions (*C24*, *Co*, and *Cal* with high phosphate contents, and *Col-0* and *Te* with low phosphate contents) were selected for detailed analysis.

### Analysis of Five Arabidopsis Accessions with Divergent PAEs

Twenty-five seedlings of accessions *C24*, *Co*, *Cal*, *Col-0*, and *Te* were grown for 24 d on 80 mL of modified MS medium (pH 7.0), solidified with 0.4% or 0.15% (w/v) agarose, and their phosphate contents were determined as described earlier.

### Measurement of Root Parameters Using Image Analysis

To characterize divergent root parameters, plants were grown for 16 d on agarose media lacking phosphate. The roots of seedlings were examined using a light microscope (AX70, Olympus, Japan) and the root regions with root hairs showing uniform maximum lengths were used for analysis. The root hair images were captured and relayed to a monitor using a charge-coupled device camera (UAFCB1, Olympus) attached to the microscope. Root hair parameters were measured using soft image analysis software (SiS GmbH, Münster, Germany) by recalling the images.

### Phosphate Uptake Experiments

For uptake studies, the plants were grown in modified  $0.5\times$  Hoagland (Hoagland and Arnon, 1938) solution in which ammonium nitrate was replaced with potassium nitrate. The phosphate sufficient and phosphate deficient media contained 500 and  $2.5 \mu\text{M}$   $\text{KH}_2\text{PO}_4$ , respectively. Surface sterilized seeds of the different Arabidopsis accessions were soaked over night in 0.08% (w/v) agarose solution. An approximate 100 seeds were sown on each  $300\text{-}\mu\text{m}$  nylon mesh ( $2 \times 2 \text{ cm}$ ). Seedlings established on the mesh were floated on the surface of the medium and were incubated with constant shaking (20 rpm) at 20°C until the experiments were performed (7–8 d). Under these conditions the roots of the seedlings grew through the openings of the nylon screen into the nutrient solution. At this developmental stage seedlings have a well-developed main root, but no lateral roots.

Phosphate uptake was measured according to Honda et al. (1998) with minor modifications. Appropriate conditions for uptake and removal of externally adsorbed phosphate were determined before uptake experiments were performed. First the optimal duration of uptake was determined by allowing the plants to absorb phosphate for different intervals. For at least 2 h phosphate uptake was linear (data not shown). For this reason, uptake time was set to 1 h. Second the duration for the removal of externally adsorbed phosphate was determined: After washing with PI buffer ( $0.1 \text{ mM CaCl}_2$ ,  $5 \text{ mM MES}$  [2-(N-morpholino)ethanesulfonic acid], pH 5.75, and  $\text{KH}_2\text{PO}_4$  added in the same concentration as in uptake solution), 8-d-old seedlings (or 7-d-old in case of 2-(N-morpholino)ethanesulfonic acid-sufficient medium) were allowed to absorb  $^{32}\text{P}$ -labeled phosphate for 15 min. These seedlings were then washed twice with ice-cold PII buffer ( $0.1 \text{ mM CaCl}_2$ ,  $5 \text{ mM MES}$ , pH 5.75, and  $1 \text{ mM KH}_2\text{PO}_4$ ) and incubated in 100 mL of PII buffer for different intervals. Incubation in ice-cold PII buffer for up to 6 h showed that minimal-labeled phosphate desorption occurred after 30 min. For experimental convenience the duration of externally adsorbed phosphate desorption was set to 2 h.

The roots of the 7- to 8-d-old seedlings were rinsed with deionized water and incubated twice for 1 h with an excess volume of PI buffer supplemented with  $\text{KH}_2\text{PO}_4$  at the same concentration as it was used in the following step ( $2.5\text{--}100 \mu\text{M}$ ) to minimize the contamination of Pi from the

solution culture. After this preincubation, the roots were carefully immersed in 7.0 mL of uptake solution (in a 12-well tissue culture plate) PI buffer, with  $\text{KH}_2^{32}\text{PO}_4$  (ICN, Irvine, CA). Pi absorption was allowed for 1 h. The plants on the mesh were transferred into 100 mL of ice-cold PII buffer for 2 h to desorb nonincorporated  $^{32}\text{P}$ . After the wash the plants were blotted dry between tissue paper and fresh weights were measured. The plants were then dried overnight in scintillation vials in a drier at 60°C. After drying, 2 mL of scintillation liquid (Readysafe liquid scintillation cocktail, Beckman Instruments, Fullerton, CA) was added and the incorporated radioactivity was measured by a liquid scintillation counter (Beckman Instruments).

The root lengths were determined by growing seedlings separately under similar conditions. Phosphate uptake was calculated as  $\text{nmol phosphate h}^{-1} \text{ cm}^{-1}$  root length and  $\text{nmol phosphate h}^{-1} \text{ g}^{-1}$  fresh mass plants.

For the determination of  $C_{min}$ , the seedlings were grown aseptically in Weck tissue culture vessels on nylon meshes prepared as described for the uptake experiments. The meshes (three meshes in each Weck vessel) were floated on the surface of 200 mL of the modified sterile MS medium lacking phosphate and supplemented with 1% (w/v) Suc. These seedlings were incubated at 20°C for 10 d. Ten-day-old seedlings were rinsed in 500  $\mu\text{M}$   $\text{CaCl}_2$ , blotted dry, and placed on 7.0 mL of solution containing 0.5  $\mu\text{M}$   $^{32}\text{P}$ -labeled  $\text{KH}_2\text{PO}_4$ . Samples were taken from the medium every hour for the first 3 h and every 2 h thereafter, for up to 9 h. Most of the phosphate (90%) was taken up in the first 3 h and thereafter uptake was reduced. The  $C_{min}$  was determined as the concentration that remained in the solution after 7 h.

### pH Change Measurement

To determine the role of acidification in HA mobilization, the pH changes in the growth medium were measured. For pH measurements, the plants were grown under sterile conditions on modified MS medium without phosphate. An approximate 300 seedlings from each accession were germinated in 200 mL of MS medium on three nylon mesh (pH was adjusted to 5.7 in the unbuffered medium before autoclaving; after autoclaving the pH was 4.76). After 10 d of growth the plants were removed, dried, and weighed. The pH of the medium was determined using a pH meter. As a control the pH of the medium without plants was also determined after 10 d of exposure to identical conditions.

### Collection of Exudates and Organic Acid Measurements

For the collection of root exudates, plants were grown in 0.5× modified Hoagland solution in the same way as for the uptake studies. The phosphate-sufficient media contained 500 and 10  $\mu\text{M}$   $\text{KH}_2\text{PO}_4$ , respectively. After 8 d of growth, seedlings were rinsed in deionized water and transferred to 12-well tissue culture plates containing 7.0 mL of deionized water per well. Meshes containing 100 seedlings per mesh were placed on wells in such a way that the roots were com-

pletely immersed in water. After 1-h incubations at room temperature the plants were removed and the exudate solutions were collected, frozen, lyophilized, and resuspended in 1.4 mL of deionized water. Fresh and dry masses of the plants were determined. The root exudates were analyzed using an anion-exchange chromatography system (DX 500, Dionex, Sunnyvale, CA) with a conductivity detector. The anions loaded on the anion-exchange column (Ionpac AS11 Analytical Column) were eluted using a solution gradient of NaOH (supplemented with methanol). The organic acid peaks were identified through retention time and were quantified according to the calibration of standard solutions.

### Statistical Analysis

Analysis of variance was performed with the procedure GLM (General Linear model, type III) of SAS Release 6.12 (SAS-Institute Inc., Cary, NC). In those cases where ANOVA indicated a significant effect of the accession on the tested variable, means were compared by LSD test. Significant different means ( $P \leq 0.05$ ) were marked with different letters in the tables or stated as significant in the text.

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