A Transgene Encoding a Plasma Membrane H^+-ATPase That Confers Acid Resistance in *Arabidopsis thaliana* Seedlings

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ABSTRACT

Proton pumps (H^+-ATPases) are the primary active transport systems in the plasma membrane of higher plant cells. These enzymes are encoded by a large gene family expressed throughout the plant, with specific isoforms directed to various specialized cells. While their involvement in membrane energetics has been suggested by a large body of biochemical and physiological studies, a genetic analysis of their role in plants has not yet been performed. We report here that mutant *Arabidopsis thaliana* plants containing a phloem-specific transgene encoding a plasma membrane H^+-ATPase with an altered carboxy terminus show improved growth at low pH during seedling development. These observations provide the first genetic evidence for a role of the plasma membrane H^+-ATPase in cytoplasmic pH homeostasis in plants.

IN fungi and higher plants, proton pumps provide the primary active transport system at the plasma membrane (reviewed in Goffeau and Slayman 1981; Serrano 1989; Sussman 1994; Michelet and Boutry 1995). These enzymes belong to a large family of cation pumps that generate ion gradients across the plasma membrane (Møller *et al.* 1996). By coupling ATP hydrolysis with the translocation of a proton, the H^+-ATPase enzyme generates electrical and pH gradients that mediate the transport of nutrients and metabolites at the cellular, tissue, and organ levels. In addition to a role in transport, the plant proton pump is thought to be involved in signal transduction and responses to the environment. For example, the plasma membrane H^+-ATPase has been implicated as playing a regulatory role in processes such as cell division, cell wall extension, and cell elongation (Cleland 1987; Senn and Goldsmith 1988; Serrano 1989; Hager *et al.* 1991; Kim and Kaufman 1995; Barbier-Brygoo *et al.* 1996).

The biological role of the plasma membrane H^+-ATPase in higher plants has been inferred from genetic studies performed with a similar enzyme in yeast (Cid *et al.* 1987; McCusker *et al.* 1987; Nakamoto *et al.* 1991), and from heterologous expression studies in which plant H^+-ATPases are expressed in yeast (Villalba *et al.* 1992; Palmgren and Christensen 1993; Palmgren and Christensen 1994; Morsomme *et al.* 1996). In *Saccharomyces cerevisiae*, two genes code for the plasma membrane H^+-ATPase (Serrano *et al.* 1986; Schlesser *et al.* 1988), but only one, PMA1, is constitutively expressed and required for growth (Serrano *et al.* 1986). This relatively simple circumstance has facilitated many physiological and cloning studies of the plant ATPase in the fungi, but the models derived from these approaches have been difficult to test *in planta*.

Gene cloning and sequencing studies in Arabidopsis and other higher plants has demonstrated that the plant plasma membrane H^+-ATPase is encoded by a large gene family containing nine or more members (Ewing and Bennet 1994; Harper *et al.* 1994; Sussman 1994). Promoter studies using reporter genes and direct measurements of mRNA levels indicate that individual gene isoforms are differentially expressed in a tissue-specific manner (Harper *et al.* 1990, 1994; DeWitt *et al.* 1991; Houlne and Boutry 1994). For example, one isoform has been found to be predominantly expressed within the plasma membrane of phloem companion cells, a specialized tissue involved in the long distance transport of sugars, nutrients and hormones (DeWitt *et al.* 1991; DeWitt and Sussman 1995). The energy to drive this active process has long been thought to be derived from the proton pump (H^+-ATPase), and not surprisingly, immunocytochemical localization studies demonstrate that the companion cell contains one of the highest concentrations of the pump in the plant (Villalba *et al.* 1991; DeWitt and Sussman 1995).

In yeast, the plasma membrane H^+-ATPase is activated *in vivo* by glucose metabolism, and deletion analysis has shown the C terminus of the enzyme to be involved in this regulation (Palmgren *et al.* 1990; Portillo *et al.* 1991). Moreover, single point mutations in the C terminus of the H^+-ATPase result in increased proton pumping that permits yeast to grow at low pH (Morsomme *et al.* 1996). Using a yeast expression system for structure-function studies of the Arabidopsis H^+-ATPase, Palmgren and coworkers have shown that deletion of amino

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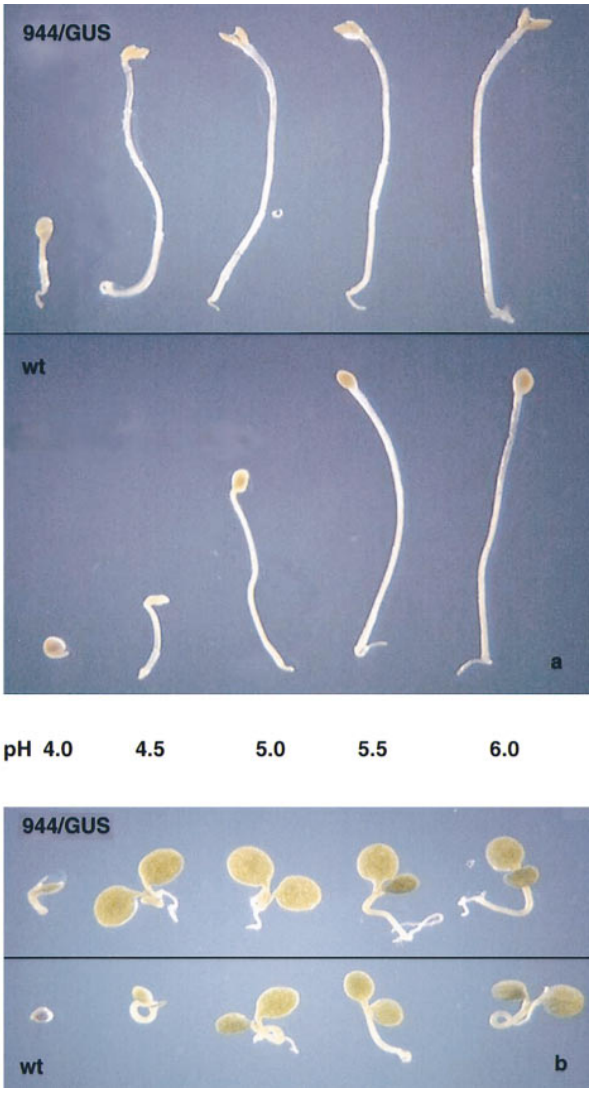

Figure 1.—Wild-type (Bensheim) and 944/GUS seedlings grown on 3 mm DMG at different pHs. (a) Dark-grown plants, and (b) light-grown plants, 7 days after germination.

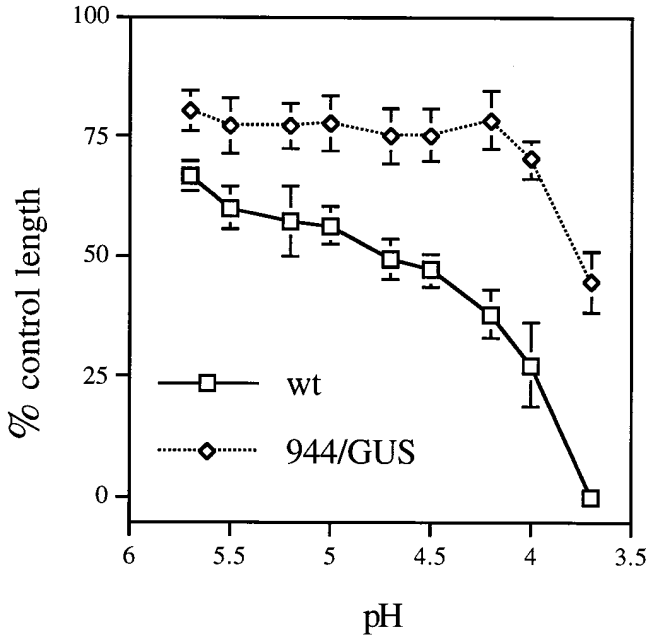

Figure 2.—pH dependence of dark-grown hypocotyl elongation. Wild-type (Bensheim) and 944/GUS transgenic seedlings were germinated and grown on 3 mm DMG at different pHs. Hypocotyl lengths of treated seedlings were compared with seedlings grown on 5 mm MES (pH 5.7). The data shown represent at least 100 seedlings per treatment ±SE.

acid residues in the C terminus of the plant pump results in a constitutively activated H^+-ATPase (Palmgren and Christensen 1993; Regenberg *et al.* 1995).

To study how the C-terminal region of the plasma membrane H^+-ATPase is involved in signal transduction and membrane transport processes in plants, we have generated mutant Arabidopsis that express an H^+-ATPase (AHA3) with a disrupted C terminus. This ATPase isoform was previously shown to be localized to the plasma membrane of companion cells (DeWitt *et al.* 1995, 1996). We show here that Arabidopsis seedlings expressing AHA3 with an altered C terminus, have improved growth at low pH.

MATERIALS AND METHODS

Plant materials and growth conditions: Arabidopsis mutant and wild-type seeds were surface sterilized in 70% (v/v) ETOH/0.1% (v/v) Triton X-100 for 5 min, rinsed 2× with 95% (v/v) ETOH and dried on sterile Whatmann paper. Sterile seeds were plated on 0.5× Murishige and Skoog media (Gibco BRL, Grand Island NY), no sucrose, with either 5 mm MES (pH 5.7 ± 0.1) or 3 mm 3,3-dimethylglutaric (DMG; Sigma, St. Louis). The pH of the medium was titrated, as indicated in Figures 1–3, with 1 N KOH prior to the addition of Agar to 1% (Sigma).

To synchronize germination, plated seeds were kept in the dark at 4° for 3–4 days, then treated with red light (30 μmol m^{-2} s^{-1}). Seeds were then transferred to either dark or light (45 μmol m^{-2} s^{-1}) conditions for 7 days. Dark-grown seedlings were measured to the nearest millimeter with a ruler. To control for differences in germination and elongation rates in the various plant lines, each experiment included growth on 5 mm MES plates (pH 5.7). The inhibition of hypocotyl elongation for each treatment is expressed as the percentage of hypocotyl length of treated seedlings compared to control seedlings.

Genetic techniques: The epitope-tagged plants and other controls were constructed as described in (DeWitt and Sussman 1995). Plants containing the transgenes were crossed with wild-type plants of the same ecotype. The F_1 progeny were scored for hypocotyl elongation in the various media, then transferred to soil and allowed to self. The F_2 progeny were similarly scored. The measurement of beta-glucuronidase (GUS) was done as described in Bleecker and Patterson (1997).

RESULTS

Previously, we described the use of epitope tagging as a means of identifying the cellular and subcellular localization of individual members within the *AHA* gene

family (DeWitt and Sussman 1995; DeWitt *et al.* 1996). The coding regions for two well-characterized 21 amino-acid epitopes from the c-*myc* mammalian oncogene and the influenza hemagglutinin (HA) proteins (Kolodziej and Young 1991) were separately fused to a 12-kb genomic clone of *AHA3*. The *AHA3* clone contains 4 kb upstream of the *AHA3* gene, including the native promoter, and 2.5 kb of 3′ flanking sequence. The epitope tags were inserted so that they would be expressed at amino acid residues 904 and 944 in the 150-amino-acid-long carboxy terminus of AHA3. This portion of AHA3 is hydrophilic and predicted to be cytoplasmically located, based on studies of the yeast H^+-ATPase (Davis and Hammes 1989; Monk *et al.* 1991). Additionally, in separate experiments, the 550-amino-acid-long bacterial protein, beta-glucuronidase (GUS) was also inserted into the 904 and 944 locations of AHA3. After transformation, all lines were selected and maintained by resistance to kanamycin (DeWitt *et al.* 1991).

Indirect immunofluorescence microscopy and Western blot analyses using antibodies directed against c-*myc*, HA or GUS epitopes demonstrated that transgenic plants containing these epitope-tagged transgenes produced fusion proteins that were targeted to the plasma membrane (DeWitt *et al.* 1996), and were expressed predominantly within companion cells of the phloem (DeWitt and Sussman 1995).

To determine whether the expression of these transgenes altered plant growth or development, we grew the transformants under a variety of physiological conditions, including assays comparing wild-type and mutant responses to phytohormones, light and gravity, and osmotic and salt stresses. In these, and other assays, the only significant difference from wild-type growth was noted when the pH of the medium was changed. The "normal" pH of our growth medium (1× Murashige-Skoog) is 5.7. To assay seedling growth at low pH, the media were buffered with a nonmetabolizable, weak DMG (Greenberg and Goldschmidt 1989). The difference in pH susceptibility as observed in both darkness and in light is shown in Figure 1. In this experiment, wild-type and mutant seeds were germinated and grown on petri plates containing 3 mm DMG with pH adjusted to 4.0, 4.5, 5.0, 5.5 or 6.0 using KOH. We observed that seeds from the transgenic lines were better able to tolerate the decreased pH, compared to wild type. To quantify this observation, we measured the hypocotyl lengths of seedlings germinated in the dark, in media containing a variety of pHs ranging from 3.7 to 5.7 (Figure 2). Wild-type growth was significantly reduced below pH 5, whereas with the transgenic line 944/GUS, a significant reduction in hypocotyl elongation was noted only at pHs below 4.0. The shape of these curves implies that the mutant is able to tolerate a tenfold higher concentration of external protons.

In order to determine whether the difference in growth at low pH is due to an altered H^+-ATPase

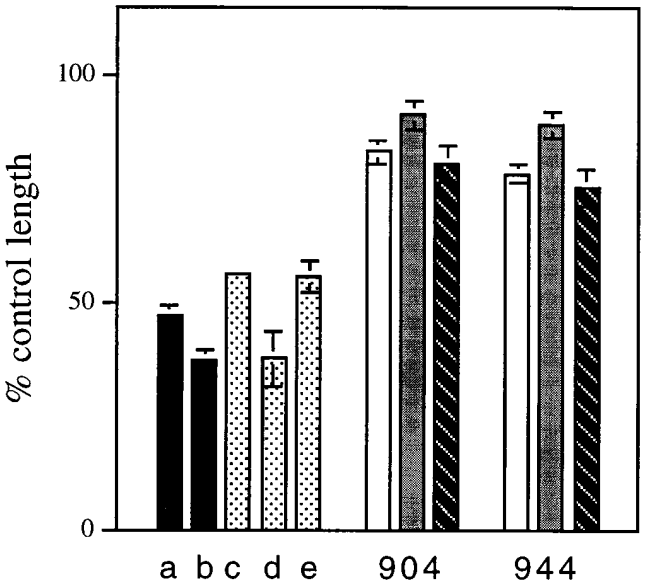

Figure 3.—Inhibition of hypocotyl elongation on low pH media. The inhibition of wild-type (a) ecotype Bensheim and (b) ecotype LER, and transgenic seedlings grown on 3 mm DMG (pH 4.7). Hypocotyl lengths of treated seedlings were compared with seedlings grown on 5 mm MES (pH 5.7). The (c) control represents the average from twelve independent lines for plants containing the 12-kb *AHA3* genomic insert with no modification of the C terminus. The 5′ control (d) contains the *AHA3* promoter alone fused to GUS, in the LER background (5 independent lines). The 3′ (e) control contains 3 kb of the 12-kb insert downstream of *AHA3* (6 independent lines). Bars labeled 904 and 944 denote the C-terminal epitopes c-*myc* (□), HA (■) and GUS (▨) at amino acid position 904 or 944 of the *AHA3* gene. The data shown represent at least 100 seedlings per treatment ±SE.

transgene, rather than an additional copy of the gene, we generated transgenic lines containing the *AHA3* transgene with no modifications. Additionally, recombinant plants with only the 5′ (4-kb), or the 3′ (3-kb) portions of the *AHA3* clone were generated to test whether the regions upstream or downstream of the *AHA3* gene contained coding sequences that improved growth at low pH. The acid-resistant phenotype was scored for all of these genotypes using the hypocotyl length assay with 3 mm DMG medium buffered to pH 4.7. The results for the lines assayed are presented in Table 1, and are summarized in Figure 3. As shown in Table 1 and Figure 3, the hypocotyl length of the Bensheim ecotype (used as the starting material for all transgenic lines) was inhibited by *ca.* 50% at pH 4.7. The transgenic plants with disruptions in the C terminus of the *AHA3* transgene show a range of effects, from 25% growth reduction (line 471-208; 904/c-*myc*) to none (line 457A; 904/HA). The average of at least two lines per construct is shown in Figure 3, except for 904/HA and 944/GUS for which only one line was assayed. As observed for wild-type control plants, all of the transgenic seedlings containing an unmodified H^+-ATPase transgene showed *ca.* 50% growth reductions. In addi-

TABLE 1

Hypocotyl elongation at low and normal pH

Line	Description	DMG (pH 4.7)	MS (pH 5.7)	Control (%)
Bensheim	wt	5.5 ± 1.9	11.7 ± 2.0	47
LER	wt	3.4 ± 1.8	10.1 ± 1.7	34
465 7305	904 c-*myc*	10.9 ± 1.5	13.1 ± 1.7	83
471 205	944 c-*myc*	9.8 ± 1.1	12.3 ± 1.3	80
471 208	944 c-*myc*	10.9 ± 1.4	14.6 ± 2.0	75
471 209	944 c-*myc*	11.4 ± 2.4	14.5 ± 3.3	78
457 A	904 HA	12.2 ± 1.2	11.9 ± 2.6	102
457 100	904 HA	10.9 ± 1.5	13.7 ± 1.2	80
457 101	904 HA	10.7 ± 1.9	11.8 ± 2.3	90
452 104	944 HA	11.9 ± 1.0	12.5 ± 2.2	95
452 105	944 HA	10.6 ± 2.1	12.8 ± 1.9	83
475 A1	904 GUS	10.9 ± 1.7	14.5 ± 3.1	75
475 B1	904 GUS	10.1 ± 1.6	11.9 ± 2.5	85
487	944 GUS	10.6 ± 2.3	14.1 ± 1.8	75
482	904 GUS	11.1 ± 1.4	14.5 ± 2.1	77
pB3s 1	AHA3	7.8 ± 0.9	14.0 ± 1.4	56
pB3s 2	AHA3	7.9 ± 1.5	14.1 ± 1.6	56
pB3s 3	AHA3	7.1 ± 1.0	13.5 ± 1.5	53
pB3s 4	AHA3	7.9 ± 1.8	14.2 ± 1.1	56
pB3s 5	AHA3	8.3 ± 1.4	16.1 ± 1.7	52
pB3s 6	AHA3	8.6 ± 2.2	13.8 ± 1.6	62
pB3s 7	AHA3	7.4 ± 1.5	12.5 ± 1.2	56
pB3s 8	AHA3	7.4 ± 1.1	12.7 ± 1.2	58
pB3s 9	AHA3	7.0 ± 1.8	11.8 ± 2.4	59
pB3s10	AHA3	7.2 ± 1.4	12.4 ± 1.4	58
pB3s11	AHA3	7.0 ± 1.4	13.7 ± 1.2	51
pB3s12	AHA3	7.2 ± 1.1	13.1 ± 1.2	55
pB3xs 1	3′ end	7.6 ± 1.4	12.3 ± 1.4	61
pB3xs 2	3′ end	7.6 ± 1.3	14.2 ± 1.5	54
pB3xs 3	3′ end	7.4 ± 1.6	13.5 ± 1.4	55
pB3xs 4	3′ end	7.7 ± 0.9	13.2 ± 1.1	58
pB3xs 5	3′ end	6.3 ± 1.7	11.6 ± 1.3	54
pB3xs 6	3′ end	6.1 ± 1.6	12.3 ± 1.7	50
3P B10	5′ GUS	4.2 ± 1.9	13.1 ± 1.7	32
3P C6	5′ GUS	3.2 ± 1.7	11.4 ± 1.5	28
3P B8	5′ GUS	3.2 ± 1.4	11.3 ± 1.8	28
3P G6	5′ GUS	5.5 ± 1.9	12.9 ± 2.1	43
3P H6	5′ GUS	3.5 ± 1.5	12.3 ± 1.5	28

Hypocotyl lengths of wild-type and transgenic seedlings grown on 3 mm DMG (pH 4.7) and 5 mm MES (pH 5.7). The lines are as described in Figure 3. The data shown represent at least 100 seedlings per treatment ±SE. The seedlings grown on MES at pH 5.7 are considered control plants. wt, wild type.

tion to the controls listed in Table 1, eleven nonrelated transgenic lines were assayed for growth at low pH, including a line in which the GUS gene is driven by the CaMV 35S promoter. All of these additional lines responded similarly to wild-type seedlings at low pH (data not shown). These results suggest that the acid resistant phenotype is conferred by the modification of the C terminus, rather than just an extra copy of the *AHA3* gene or a transformation artifact.

If the acid resistant phenotype is caused by expression of the transgene containing a disruption of the AHA3 C terminus, it should segregate as an inherited single gene. To test this, we crossed six lines (expressing a variety of epitopes, at both 904 and 944 positions) with wild type, and then tested them for acid resistance in both the F_1 and F_2 generations (Table 2). In each case, the F_1 seeds were all resistant, but in the F_2 generation, three-fourths were resistant, consistent with a dominant effect of the transgene. The cosegregation of the transgene with acid resistance was tested by assaying for GUS expression in F_2 families of lines with GUS inserted in the C terminus of AHA3. Two independent lines, 904/GUS and 944/GUS, were assayed, and both showed the segregation of GUS expression with improved hypocotyl elongation at low pH (Table 2). These results are consistent with the conclusion that any plant containing a single copy of the transgene is significantly more resistant to acid conditions than wild type.

TABLE 2

Segregation of the acid resistant phenotype

Cross		Resistant	Sensitive	χ^2
904/c-*myc* × wt	F_1	37	0	—
	F_2	24	6	0.4
	F_2	42	14	0.1
944/c-*myc* × wt	F_1	54	0	—
	F_2	40	8	1.8
	F_2	43	16	
904/HA × wt	F_1	37	0	—
	F_2	29	5	NS
	F_2	73	36	3.7
944/HA × wt	F_1	38	0	—
	F_2	36	11	0.4
904/GUS × wt	F_1	72	0	—
	F_2	103	31	0.3
944/GUS × wt	F_1	134	0	—
	F_2	47	9	2.3
		Resistant (GUS expression)	Sensitive (GUS expression)	G_{adj}
904/GUS × wt	F_2	46 (44)	13 (1)	39.2
944/GUS × wt	F_2	41 (38)	13 (1)	26.0

Homozygous plants containing the various *AHA3* epitope tagged transgenes were crossed with wild-type (Bensheim) plants. F_1 and F_2 plants were scored for dark-grown hypocotyl elongation on 3 mm DMG, pH 4.7, after 7 days. In some instances, progeny from two F_2 families were assayed for growth at low pH. Seedlings were scored as resistant with long hypocotyls (>8 mm), and sensitive with short hypocotyls (<8 mm). Additionally, F_2 seedlings from the GUS-tagged lines were histochemically tested for GUS activity, using the chromogenic substrate X-GLUC. Acid-resistant and -sensitive seedlings were scored for GUS expression. The *G*-test (Sokal and Rohlf 1969) values for the cosegregation of the GUS tag with acid-resistance yield $P >> 0.995$. wt, wild type; NS, not significant.

DISCUSSION

Earlier studies comparing the structure and function of genetically altered H^+-ATPase proteins in yeast established that the carboxy-terminal domain of the fungal proton pump is an autoinhibitory domain (Portillo *et al.* 1991; Sekler *et al.* 1994). Similarly, when plant H^+-ATPase genes were expressed in yeast and tested biochemically, it was observed that changes in the carboxy terminus were associated with increases in catalytic activity (Villalba *et al.* 1992; Palmgren and Christensen 1993, 1994; Morsomme *et al.* 1996). These changes were not due to differences in expression, biogenesis or protein stability. Rather, they reflected true catalytic differences in affinity for substrates (K_m) or in the rate at which the enzyme turns over during the catalytic cycle (V_{max}). *In vitro* biochemical studies with the plant enzyme, in which the carboxy terminus was proteolytically cleaved, further confirmed that the carboxy terminus down-regulates the catalytic activity of the H^+-ATPase (Rasi-Caldognov *et al.* 1993).

Despite the numerous *in vitro* experiments with purified plasma membranes, or with the enzyme expressed in yeast, direct *in situ* evidence for this phenomenon within a higher plant has not been reported. In this article we have presented the first *in planta* evidence that the carboxy terminus is indeed acting as an autoinhibitory domain within the intact plant. This conclusion is based on our observation that plants containing a variety of transgenes with a carboxy-terminal disruption, all show increased resistance to growth inhibition at low pH.

It is striking to note that this transgene, which behaves genetically as a single dominant gene, encodes an enzyme which is predominantly expressed only in companion cells of the phloem. This raises the interesting question of why such an expression pattern should cause resistance of the overall plant to growth at acid pH. A likely answer is that in these young seedlings lacking a cuticle, the phloem may be a primary target of low pH. It is known that the phloem sap is alkaline at pH 8.0 or higher (Vreugdenhill and Koot-Gronsveld 1989). Thus, the phloem may be more sensitive to the steeper difference in pH across the plasma membrane than other cells within the stem, leaves and roots. Furthermore, it is likely that a deleterious change in the phloem pH could result in an overall change in growth rates, since the phloem mediates the long distance transport of essential sugars, nutrients and hormones. In the

transgenic plants, a modified proton pump expressed in the phloem appears to allow these cells to maintain proper function at low pHs. It is likely that the modified *AHA3* gene produces an unregulated pump, and, as in yeast with point mutations in the carboxy terminus (Morsomme *et al.* 1996), growth is maintained at low pH. However, further studies will be needed to test our hypothesis that the phenotype is caused by post-translational changes in catalytic activity, rather than changes in expression, stability, or targeting of the AHA3-tagged mRNA or protein.

A final word should be made about expression of plasma membrane transporters as transgenes. Although we have attempted to overexpress and underexpress H^+-ATPase genes in Arabidopsis and tobacco using the 35S promoter with a variety of H^+-ATPase sense and antisense constructs, we have never been able to generate plants in which the H^+-ATPase concentration is noticeably altered or which show an altered phenotype. It is possible that we were successful in the experiments reported here because a natural promoter, which directed expression to a single cell type, was used. For enzymes that perform essential functions, it may be important to direct expression of transgenes to precise locations and developmental stages that are not deleterious to growth and development.

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