

An *Arabidopsis thaliana* Plasma Membrane Proton Pump Is Essential for Pollen Development

Whitney R. Robertson,* Katherine Clark,[†] Jeffery C. Young[†] and Michael R. Sussman*^{‡,1}

*Program in Cellular and Molecular Biology and [†]Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706 and

[‡]Department of Biology, Western Washington University, Bellingham, Washington 98225

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ABSTRACT

The plasma membrane proton pump (H^+ -ATPase) found in plants and fungi is a P-type ATPase with a polypeptide sequence, structure, and *in vivo* function similar to the mammalian sodium pump (Na^+ , K^+ -ATPase). Despite its hypothetical importance for generating and maintaining the proton motive force that energizes the carriers and channels that underlie plant nutrition, genetic evidence for such a central function has not yet been reported. Using a reverse genetic approach for investigating each of the 11 isoforms in the *Arabidopsis* H^+ -ATPase (*AHA*) gene family, we found that one member, *AHA3*, is essential for pollen formation. A causative role for *AHA3* in male gametogenesis was proven by complementation with a normal transgenic gene and rescue of the mutant phenotype back to wild type. We also investigated the requirement for phosphorylation of the penultimate threonine, which is found in most members of the *AHA* family and is thought to be involved in regulating catalytic activity. We demonstrated that a T948D mutant form of the *AHA3* gene rescues the mutant phenotype in knockout *AHA3* plants, but T948A does not, providing the first *in planta* evidence in support of the model in which phosphorylation of this amino acid is essential.

THE plasma membrane proton ATPase (H^+ -ATPase) of plants and fungi creates a proton motive force, which is used directly by most secondary transporters to mediate the movement of solutes into and out of the cell (MORSOMME and BOUTRY 2000; PALMGREN 2001). In *Arabidopsis*, the electrochemical potential has been recorded at very negative levels, such as -230 mV, while the corresponding protein in animal cells, the Na^+ , K^+ -ATPase, typically generates membrane potentials of only ~ -100 mV (HIRSCH *et al.* 1998). This potential, together with a chemical gradient of protons, is thought to be essential for diverse cellular processes, including nutrient transport, cellular expansion, and osmoregulation. Thus, the proton ATPase has been hypothesized to play a crucial role in many important physiological processes.

The family of genes encoding plasma membrane H^+ -ATPases in *Arabidopsis* has 11 members (PALMGREN 2001). Expression data have implicated differing roles for family members in numerous tissues, including root layers, floral organs, and vasculature (HARPER *et al.* 1990; HOULNE and BOUTRY 1994; DEWITT and SUSSMAN 1995). These proteins are intriguing targets for genetic studies that test the hypothesis that they play important roles in plant physiology.

One family member, *AHA3*, was shown to be expressed

in companion cells of the phloem and various reproductive tissues (DEWITT *et al.* 1991; DEWITT and SUSSMAN 1995). Its role in the phloem is presumed to be the control of sugar loading for long-distance sucrose transport, a process that is critical for plant nutrition. While localization studies were useful in determining the expression pattern of *AHA3*, we wanted to more closely and directly examine the *in planta* role of this gene. To this purpose, we took a reverse genetic approach and characterized the effect of the absence of functional *AHA3* on the plant. Through transmission studies, microscopic analysis, and functional complementation, we have identified an essential role for *AHA3* in pollen development.

Knockout plants are useful for structure/function experiments, which are designed to test the roles of specific domains. Results of heterologous experiments with yeast suggested that T948, at the extreme C terminus of *AHA3*, is essential for activation of the pump. To test the hypothesis that this residue is important for functions of the pump *in planta*, we mutated T948 to either aspartate or alanine. Transgenes carrying these mutations were introduced into $+ / aha3-5$ plants. Plants containing these transgenes in an *aha3* null background were identified among the progeny and characterized. Our results indicate that the highly conserved penultimate threonine residue is not absolutely required *in planta*, but that complementation is dependent on the presence of a negative charge at this location since substitution of T with A did not functionally replace *AHA3*. However, substitution of T with D rescued *aha3* plants and con-

¹Corresponding author: Biotechnology Center, 425 Henry Mall, University of Wisconsin, Madison, WI 53706.
E-mail: msussman@facstaff.wisc.edu

ferred a growth advantage to seedlings on acidic media, presumably due to hyperactivation of *AHA3*. These results are consistent with a predicted role for this residue in regulation of activation by an as-yet-unidentified protein kinase.

MATERIALS AND METHODS

T-DNA mutant screen and identification: T-DNA mutagenized populations were screened for the presence of insertions in *AHA3* using a PCR-based strategy (KRYSAN *et al.* 1996, 1999). The sequences of primers specific for *AHA3* were 5'-CCAAATAGCCTAACGTAGTCCACCTTCAC, 5'-AATATGACTAGCACAGTAGCACCTTTACC, 5'-GAATAAGGAAGGAAGAAAACCCCAGGAG, and 5'-TCTCGTCTTCTTGGTTTTGTTTTGTAGC. The sequence of a primer specific for the T-DNA border was 5'-CATTTTATAATAACGCTGCGGACATCTAC (JL202). An individual plant containing the *aha3-1* allele was isolated from a population of 64,860 lines mutagenized with a derivative of the T-DNA vector pD991 (YOUNG *et al.* 2001). This vector carries a selectable marker gene that renders plants resistant to kanamycin. Individual plants containing the *aha3-5* allele were isolated from a population of 72,960 lines mutagenized with the pSKI15 vector. This vector carries the selectable marker *bar* gene that renders plants resistant to glufosinate, the active ingredient in the herbicide BASTA. DNA sequencing of PCR products confirmed the locations of the junctions of genomic and T-DNA sequences. Both *aha3* mutant alleles were present in the Wassilewskija (Ws) background.

Mutant transcript identification: RNA was isolated from plants heterozygous for either the *aha3-1* or the *aha3-5* allele using the QIAGEN RNeasy mini kit (Valencia, CA). cDNA was synthesized using a modified oligo(dT) primer (5'-24T + A/C/G/T) and SuperScript II reverse transcriptase (Invitrogen, San Diego). Nested primers specific to *AHA3* cDNA and to the T-DNA border sequence (primer JL202) were used to amplify PCR products corresponding to the mutant *aha3* transcripts. The cDNA-specific primers used were 5'-ACAGACTGTACCAGAGAAAACAAA and 5'-AGAGTCAAGCCTTCTCCAA CACCAGATAG (for *aha3-1*) and 5'-GGTATCGTTTTGTCTTTTGGTTATCA and 5'-CGAAGAAAACAATGCTGGAATGCT (for *aha3-5*). DNA sequencing of PCR products confirmed the location of the junction between *AHA3* cDNA and T-DNA.

Media and plant growth: Seeds were germinated on plates containing half-strength Murashige and Skoog (MS) salts, 1% (w/v) sucrose, and 0.8% (w/v) washed agar (MS plates). Seedlings were transferred to soil after 10–14 days. Plants were housed under the following growth conditions: 21°, 24 hr light (42 $\mu\text{E}/\text{m}^2/\text{sec}$) and ~30% humidity.

The genotype of individual plants was determined by PCR using *AHA3*-specific primers and primer JL202. Two *AHA3*-specific primers were used to detect the presence of the wild-type allele. An *AHA3*-specific primer and primer JL202 were used to detect the presence of the mutant allele.

To select plants containing the *aha3-1* allele, seedlings were germinated on MS plates containing 50 $\mu\text{g}/\text{ml}$ kanamycin. To select plants containing the *aha3-5* allele, seeds were sown in soil, and seedlings were subsequently sprayed with BASTA herbicide (50 $\mu\text{g}/\text{ml}$ glufosinate) at 4 and 8 days.

Determination of male gametophyte lethality: Reciprocal crosses were performed between wild-type (Ws) and *AHA3/aha3-1* or *AHA3/aha3-5* plants. Progeny were germinated on MS plates containing 50 $\mu\text{g}/\text{ml}$ kanamycin (for *aha3-1*) or 25 $\mu\text{g}/\text{ml}$ glufosinate (for *aha3-5*).

To confirm male gametophytic lethality, *AHA3/aha3-5* plants were crossed as females to *qrt1-3/qrt1-3* plants (gift from Greg-

ory Copenhaver; lab stock CLA18). The F_1 's were selfed and F_2 progeny heterozygous for *aha3-5* were selected by spraying with BASTA herbicide (50 $\mu\text{g}/\text{ml}$ glufosinate). Plants homozygous for the *qrt* mutation were identified by visualization of "quartets" of pollen grains (PREUSS *et al.* 1994).

Light microscopy and staining: To visualize mutant pollen grains, anthers from a plant heterozygous for *aha3* were collected and dabbed on a microscope slide coated with a thin layer of pollen germination media (MOULINE *et al.* 2002). Pollen grains and emerging tubes were viewed using a Nikon E400 clinical microscope. Images were recorded using a Spot Insight color digital camera (Diagnostic Instruments).

To visualize color-differentiated wild-type and mutant pollen grains, anthers were collected and squashed on a microscope slide. Alexander's stain solution, prepared according to Alexander (1969) and used at a concentration of 3 parts stain/47 parts water, was added; the sample then was covered with a coverslip and viewed using an Olympus BX60 microscope (Olympus Optical, Tokyo). Images were captured using an Olympus DP12 digital microscope camera (Olympus Optical).

Scanning electron microscopy: We prepared samples for scanning electron microscopy using tested methods (FERNANDEZ *et al.* 2000). Briefly, flowers were collected and fixed in 4% (v/v) glutaraldehyde, rinsed in 0.05 M KPO_4 , dehydrated in an ethanol series, and critical point dried using CO_2 . Samples were mounted on steel posts covered with adhesive circles (Ted Pella, Redding, CA) and sputter coated with a thin film of gold. Samples were viewed at 10,000 accelerating voltage on a Hitachi S-570 scanning electron microscope (Hitachi, Tokyo). Images were recorded digitally using a Gatan digital image capture system and digital micrograph version 2.5 software (Gatan, Pleasanton, CA).

Flower fixation and sectioning: We prepared flowers for sectioning using tested methods (FERNANDEZ *et al.* 2000). Briefly, flower buds from all ages were collected, fixed in 4% (v/v) glutaraldehyde, rinsed in 0.05 M KPO_4 , dehydrated in an ethanol series, and embedded in London Resin white medium-grade resin (EMS, Fort Washington, PA). The embedded flowers were sectioned in 2 μ increments using an ultramicrotome (RMC, Tucson, AZ). Sections were heat fixed on a microscope slide, stained with 0.05% (w/v) toluidine blue O, and viewed with bright-field optics using a Nikon E400 clinical microscope. Images were recorded using a Spot Insight color digital camera (Diagnostic Instruments).

To visualize β -glucuronidase (GUS) staining in sections from C-terminally modified *AHA3* transgenic plants, the protocol described above was followed, with the addition of one step. Prior to fixation in glutaraldehyde, samples were incubated in modified X-Gluc buffer (0.5 mg/ml X-Gluc, 50 mM KPO_4 , 2.5 mM potassium ferricyanide, and 2.5 mM potassium ferrocyanide) for at least 6 hr. To view GUS color precipitates, the sections were left unstained and observed with dark-field optics using an Olympus BX60 microscope.

Molecular complementation: A 9-kb *SpdI* fragment containing the entire regulatory and coding sequence of *AHA3* (N. D. DEWITT, unpublished results) was ligated into the binary vector pCAMBIA3300S, a spectinomycin-resistant derivative of pCAMBIA3300. This vector contains the coding sequence of the *bar* gene between the T-DNA border regions. *AHA3/aha3-1* plants (designated the T_0 generation) were transformed with *AHA3::pCAMBIA3300S* using the *Agrobacterium tumefaciens*-based floral dip method (CLOUGH and BENT 1998). Transformants (T_1) were selected in soil by spraying with BASTA (50 $\mu\text{g}/\text{ml}$ glufosinate) at 4 and 8 days postgermination. Rescued plants were identified among the T_2 plants using a PCR-based strategy. Rescued plants were defined as those homozygous for the *aha3-1* allele, but containing an *AHA3* transgene. Primers used to identify the wild-type, native *AHA3* allele were 5'-CTAACCACT

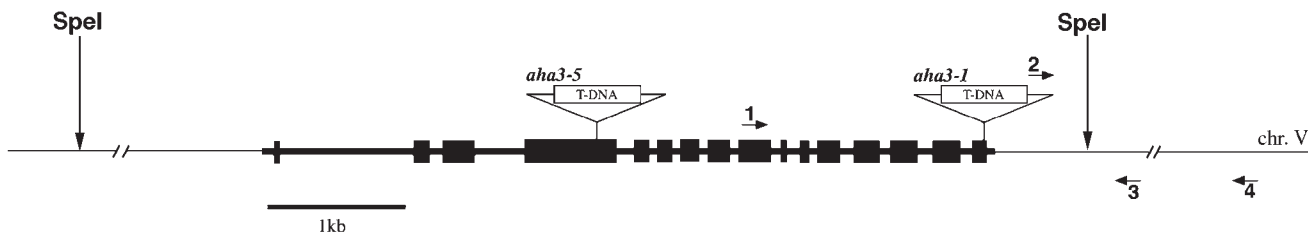


FIGURE 1.—Structures of *aha3* alleles and *AHA3* fragment for molecular complementation. In the PCR-based strategy to identify rescued *aha3-1/aha3-1* plants, primers 1 and 4 amplify a product corresponding to native *AHA3*; primers 2 and 3 amplify a product corresponding to *aha3-1*. Boxes represent exons; lines represent introns. All T-DNA borders are canonical left-border sequences. T-DNA is not drawn to scale.

AGAGCCATCTTCCAGAGAATG and 5'-AATAACCAAGAAAGTAGCGGACCAAACAC; those used to identify the *aha3-1* allele were 5'-TTATTAGTGTGGTCGTTGGGGTATCTTG and the T-DNA-specific primer, JL202.

Molecular complementation with altered *AHA3* clones: Altered clones of *AHA3* were constructed using the Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primers used to introduce the alterations for threonine/alanine substitution were 5'-GCTGGTCACTACGCCGTTTAATAAAGATTTAAC and its complement and for threonine/aspartate substitution, 5'-GCTGGTCACTACGACGTTTAATAAAGATTTAAC and its complement. The identities of the desired clones were confirmed by DNA sequencing of PCR products spanning the alteration. Clones were ligated into the binary vector pGreen0029, which carries the selectable marker *nptII* gene that confers resistance to kanamycin in transgenic plants. Plants heterozygous for *aha3-5* (designated the T₀ generation) were transformed with altered *AHA3::pGreen0029* using the *A. tumefaciens*-based floral dip method (CLOUGH and BENT 1998). T₁ seedlings were selected on MS plates containing 50 µg/ml kanamycin. Rescued mutants among the T₂ generation were identified using a PCR-based strategy. Rescued plants were defined as plants homozygous for the *aha3-5* allele, yet alive due to the presence of the *AHA3* transgene. Primers used to identify the wild-type, native *AHA3* allele were 5'-TTGTCGGTATCGTTTGTCTTTTGGTTATC and 5'-AATAACCAAGAAAGTAGCGGACCAAACAC. Primers used to identify the *aha3-5* allele were 5'-AATATGACTAGCACAGTAGCACCTTTACC and the T-DNA-specific primer JL202.

Assays of transgenic seedlings: Seedlings containing altered *AHA3* transgenes were germinated on growth media at pH 4.5, 5, or 5.5 according to methods described previously (YOUNG *et al.* 1998). After 10 days, hypocotyls of dark-grown seedlings were measured to the nearest millimeter with a ruler.

RESULTS

Identification and characterization of *aha3* alleles:

A PCR-based, reverse-genetic strategy was used to identify Arabidopsis plants containing mutant alleles of the plasma membrane transporter *AHA3* (KRYSAN *et al.* 1996). In all, five *aha3* alleles were identified from T-DNA-mutagenized populations of Arabidopsis. A diagram of the insertion sites for the alleles *aha3-1* and *aha3-5* is shown in Figure 1.

***aha3-1*:** The *AHA3* wild-type genomic sequence is ~6 kb in length and contains 16 exons. A plant containing one copy of the mutant allele *aha3-1* was isolated from a T-DNA-mutagenized population of 60,470 independently

transformed lines (YOUNG *et al.* 2001). DNA sequencing confirmed the location of T-DNA within the gene. In *aha3-1*, the T-DNA was located in the last exon, 47 bases from the stop codon. Both sides of the T-DNA insert were intact, with canonical left-border sequences facing outward (data not shown). No plants homozygous for the T-DNA insertion allele were ever found, suggesting that it was lethal for development of the embryo or one of the gametes.

To determine whether the *aha3-1* allele was expressed, RNA was isolated from whole plants heterozygous for *aha3-1*. The sequence of the corresponding cDNA confirmed the existence of the *aha3-1* transcript. The *aha3-1* transcript was identical to that of wild type, minus 65 bases from the 3' end. Starting at this site, the sequence was T-DNA encoded and extended for at least 213 bases (data not shown).

It was not determined whether an *aha3-1* protein was present. On the basis of the location of the T-DNA insertion, one can predict that if *aha3-1* protein were made, it would be identical to wild-type *AHA3* protein, minus 21 amino acids from the C terminus. At this location, the protein sequence would contain an additional 71 T-DNA-based amino acids. Therefore, any *aha3-1* protein would lack a portion of the autoinhibitory domain, which is involved in enzyme regulation (PALMGREN 2001). However, it is unknown whether this loss would result in protein instability or, if stable, an alteration in enzyme catalytic activity.

***aha3-5*:** A plant containing a copy of the mutant allele *aha3-5* was isolated from a T-DNA mutagenized population of 72,960 lines. DNA sequencing confirmed the location of the T-DNA within the fourth exon. Both sides of the T-DNA insert were intact, with canonical left-border sequences facing outward (data not shown).

To determine whether the *aha3-5* allele was expressed, RNA was isolated from plants heterozygous for *aha3-5*. The sequence of a PCR product corresponding to the *aha3-5* transcript revealed that it was just over one-third of the length of the full-length *AHA3* message. It contained 17 bases of unknown origin, followed by at least 213 bases of T-DNA-encoded sequence at the 3' end (data not shown).

TABLE 1

PCR genotyping of progeny of seedlings heterozygous for *aha3*

Parent genotype	Progeny genotype			<i>P</i>
	<i>AHA3/AHA3</i>	<i>AHA3/aha3</i>	<i>aha3/aha3</i>	
<i>AHA3/aha3-1</i>	126	121	0	0.9
<i>AHA3/aha3-5</i>	110	115	0	0.9

Due to the location of the T-DNA in the first third of *AHA3*, it was unlikely that any functional *AHA3* protein was made. On the basis of the sequence of the *aha3-5* message, any *aha3-5* protein would contain the first 353 of 949 amino acids of wild-type *AHA3* protein, followed by 71 T-DNA-encoded amino acids. The first 353 amino acids include the first four transmembrane domains, but not the pore, the large cytoplasmic loop containing the ATP-binding site or regulatory C-terminal domain.

Genetic characterization of *aha3* plants: Plants heterozygous for the *aha3-1* or *aha3-5* allele were selfed, and progeny were tested by PCR for segregation of the mutation. Selection on media containing kanamycin or BASTA was also used to analyze segregation of *aha3*, since the T-DNA contained a selectable marker gene that conferred resistance to either kanamycin (*aha3-1*) or BASTA (*aha3-5*). The observed ratios of wild-type to heterozygous plants and kanamycin sensitive (Kan^s) to kanamycin resistant (Kan^R) plants were consistent with a 1:1 ratio of segregation of the *aha3* mutation (Tables 1 and 2). This segregation ratio is characteristic of mutations that cause defects or are lethal during the haploid gametophytic phase of the plant life cycle. In addition, the segregation data indicated that no other T-DNA insertion was present in the background. One explanation for the discrepancy of the 1.3:1 ratio of BASTA^s to BASTA^R plants could be that the gene conferring resistance to BASTA was silenced in a small percentage of heterozygotes, resulting in seedling sensitivity despite the presence of the T-DNA. This silencing is a common observation (MATZKE and MATZKE 1998; KOPREK *et al.* 2001).

TABLE 2

Genotyping progeny of *aha3* mutants by selection on antibiotics or herbicide

Parent genotype	Progeny phenotype		<i>P</i>
	Sensitive	Resistant	
<i>AHA3/aha3-1</i>	82	85	0.9
<i>AHA3/aha3-5</i>	127	96	0.05

Progeny of heterozygous parents were PCR genotyped and tested for the ability to grow on selective media. Kanamycin was used (50 $\mu\text{g}/\text{ml}$) to detect the presence of *aha3-1*; BASTA herbicide (50 $\mu\text{g}/\text{ml}$) for *aha3-5*.

TABLE 3

Determination of gametophytic lethality by reciprocal crosses

Female parent genotype	Male parent genotype	Progeny	
		<i>AHA3/AHA3</i>	<i>AHA3/aha3</i>
<i>AHA3/aha3-1</i>	<i>AHA3/AHA3</i>	23	25
<i>AHA3/AHA3</i>	<i>AHA3/aha3-1</i>	52	0
<i>AHA3/aha3-5</i>	<i>AHA3/AHA3</i>	55	52
<i>AHA3/AHA3</i>	<i>AHA3/aha3-5</i>	101	0

Therefore, despite a *P*-value of 0.05, we accepted the likelihood that the 1:1 ratio of segregation of the *aha3-5* mutation was true.

***aha3* mutant plants display male gametophytic lethality:** To determine why homozygous *aha3* plants could not be found, reciprocal crosses between wild-type plants and plants heterozygous for *aha3* were performed. Seeds from individual crosses were harvested, and progeny were germinated on media containing either kanamycin or BASTA (for *aha3-1* and *aha3-5*, respectively) to test for transmission of the T-DNA. The results are shown in Table 3. Transmission of the T-DNA through the male gamete was not observed for either allele. Transmission of the T-DNA through the female gametophyte was normal for both alleles.

Cytological and morphological analysis of *aha3* pollen: Pollen grains from wild-type and $+/aha3-5$ flowers were dabbed onto a slide covered with germination media and viewed using a microscope. Figure 2A shows pollen from a wild-type flower. All pollen grains were uniformly shaped and sized. Figure 2E shows pollen from a $+/aha3-5$ flower. A mixture of full, round pollen grains and smaller, misshapen pollen grains was observed. In some instances, pollen tubes were seen emerged from full, round pollen grains, but none from the smaller, misshapen grains. We repeatedly observed a 1:1 ratio of full, round pollen grains to smaller, misshapen pollen grains.

A stain was applied to pollen grains from a $+/aha3-5$ plant to distinguish viable and nonviable pollen (ALEXANDER 1969). In Alexander's stain, viable pollen grains are purple with a green outline, and aborted pollen grains are green inside and out. The basis for this differential staining may be a pH difference in the cytoplasm between live and dead pollen (ALEXANDER 1969). Pollen grains from a wild-type plant were viewed as full, round, purple-stained grains with a green outline (Figure 2B); and, occasionally, as smaller, misshapen, green-stained grains, presumably due to a small, normal rate of wild-type pollen abortion). However, pollen grains from a $+/aha3-5$ plant were viewed as a mixture of purple grains with a green outline and green, misshapen grains (Figure 2F).

To examine the morphological differences between wild-type and mutant pollen grains more closely, pollen

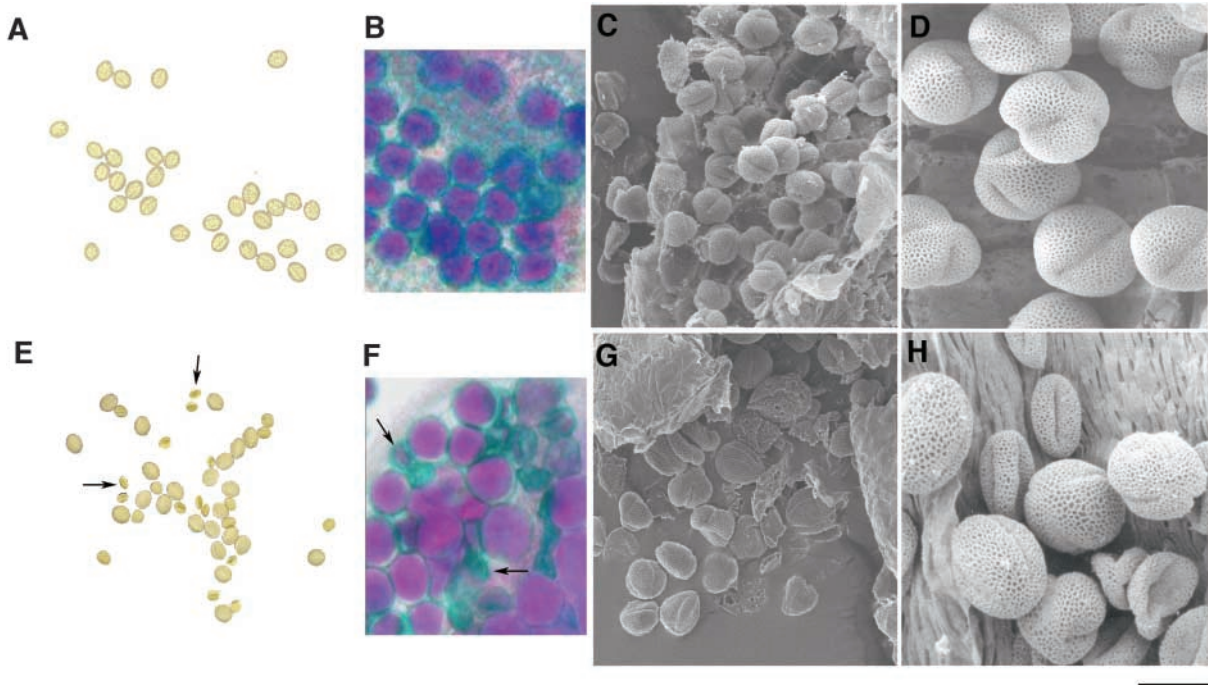


FIGURE 2.—Microspores or pollen grains from wild-type (A–D) and $+/aha3-5$ (E–H) anthers. (A and E) Pollen grains applied to a glass slide. Arrows in E indicate mutant pollen grains. (B and F) Pollen grains still attached to the anther, stained with Alexander's viability stain. Arrows in F indicate mutant pollen grains. The observed frequencies of mutant pollen grains stained with Alexander's stain were $3.2\% \pm 0.6$ in $AHA3/AHA3$ flowers and $50.9\% \pm 0.7$ in $AHA3/aha3-5$ flowers. (C and G) Immature microspores. (D and H) Mature pollen grains. Magnification of micrographs, $1000\times$; bar, $15\ \mu\text{m}$.

samples were prepared for scanning electron microscopy. Anthers and pollen grains from a spectrum of flower ages were analyzed. Microspores, or immature pollen, from young flowers were uniformly shaped and sized (Figure 2, C and G). All microspores appeared round with slight definitions along lobe segments. In some cases, a fraction of microspores from $+/aha3-5$ flowers appeared slightly smaller or collapsed. These microspores could be *aha3* microspores in the early stages of abortion. Pollen from mature wild-type or $+/aha3-5$ anthers were also compared (Figure 2, D and H). Pollen from a mature wild-type anther was uniformly full and round, whereas pollen grains from a $+/aha3-5$ anther were a mixture of shapes and sizes. Roughly half of the pollen appeared full and round, and half appeared shrunken and collapsed. Overall, our microscopic studies are consistent with the hypothesis that the smaller, misshapen pollen grains probably correspond to *aha3* haploid mutant grains.

To determine a more precise developmental stage at which half the microspores in $+/aha3-5$ plants aborted, semithin sections of fixed wild-type and $+/aha3-5$ inflorescences were viewed. Figure 3, A and E, shows sections through pollen mother cells of both wild-type and $+/aha3-5$ anthers. No differences were detected, which was expected from premeiotic cells. Figure 3, B and F, shows sections through tetrads, which correspond to the stage of pollen development immediately following

meiosis. It appeared that there were no visible differences in the morphology of the tetrads. Each product of meiosis, in both wild-type and $+/aha3-5$ anthers, appeared identical. This indicated that the abortion associated with half of the members of each tetrad, *i.e.*, the *aha3* microspores, was not yet apparent. Figure 3, C and G, shows sections through vacuolated microspores. Again, no differences between microspores from wild-type or $+/aha3-5$ anthers or among microspores from $+/aha3-5$ anthers were detectable. Differences between microspores from wild-type or $+/aha3-5$ anthers or among microspores from $+/aha3-5$ anthers were not obvious until mature pollen sections were viewed (Figure 3, D and H). These sections very clearly revealed that roughly half of the pollen grains from $+/aha3-5$ flowers were misshapen and empty. The other half were full-sized and stained throughout, identical to pollen grains from wild-type anthers. Taken together, these results indicated that *aha3* pollen abortion occurred between the stages corresponding to vacuolated microspores and mature pollen.

Genetic confirmation of *aha3* male gametophytic lethality: *qrt* mutants are pollen mutants in which the separation of the four products of meiosis is defective, resulting in "quartets" of pollen grains (PREUSS *et al.* 1994). By expressing a pollen lethal mutation in a *qrt* background, it is possible to use tetrad analysis to examine the products of meiosis. A plant heterozygous for

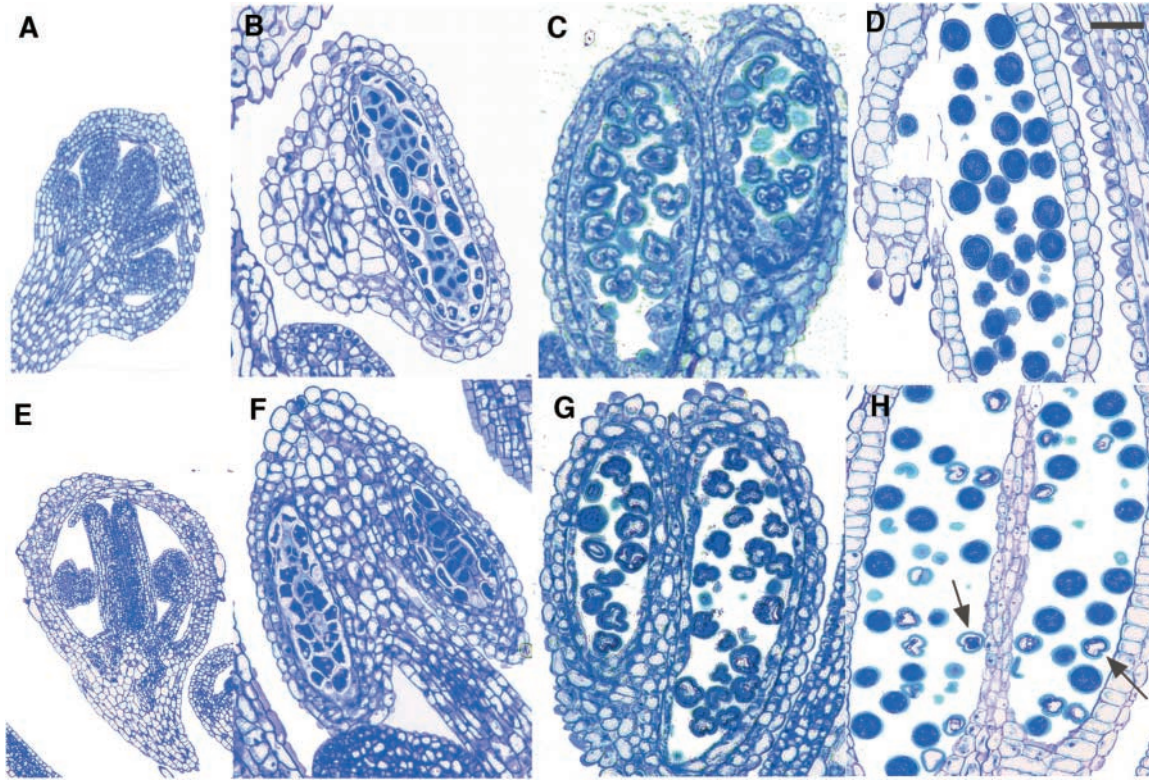


FIGURE 3.—Cross sections of wild-type (A–D) and $+/aha3-5$ (E–H) anthers. (A and E) Pollen mother cells. (B and F) Tetrads. (C and G) Vacuolate microscores. (D and H) Mature pollen. Arrows in H point to $aha3-5$ pollen grains. Bar, 30 μm .

$aha3-5$ was crossed as a female to a qrt/qrt plant. $+/aha3-5$, qrt/qrt plants were identified in the F_2 generation, and pollen grains were viewed microscopically. “Quartets” of mature pollen grains showed two normal and two aborted microspores (Figure 4A). For comparison, the smaller box in Figure 4A depicts a $+/+$, qrt/qrt pollen quartet. Figure 4B shows quartets stained with Alexander’s stain.

AHA3 expression in male gametophyte tissues: Previous work using immunolocalization of epitope-tagged AHA3 in transgenic plants or histochemical analysis of plants containing AHA3 reporter constructs depicted

AHA3 localization in a variety of plant tissues, including vasculature and reproductive tissues (DEWITT *et al.* 1991; DEWITT and SUSSMAN 1995). However, depictions of AHA3 expression in male gametophyte tissues were limited to mature pollen. No reports of AHA3 expression in younger male tissues were made. Therefore, to explain the appearance of an $aha3$ -related phenotype during early microspore development, it was necessary to determine whether AHA3 was expressed in younger, male tissues.

Flowers from plants containing a modified AHA3 transgene, in which the reporter protein GUS was fused to the C terminus of AHA3, were examined. The flowers were incubated with GUS substrate, fixed, and sectioned using an ultramicrotome. Sections were viewed with dark-field optics to visualize GUS color precipitates. GUS expression was seen in several stages of microspore development (Figure 5). Figure 5, A–C, shows the lack of expression of AHA3 in pollen mother cells, tetrads, and prevacuolate microspores, respectively. Figure 5, D–F, shows AHA3 expression in the early developmental stages corresponding to early vacuolate microspores, vacuolate microspores, and microspores undergoing the first mitotic division (REGAN and MOFFATT 1990). The level of expression was strongest in the early vacuolate microspore stage and diminished with each stage. In mitotically active pollen, staining was nearly absent (Figure 5G). In mature pollen, expression was completely absent (Figure 5H). The pattern of expression was sig-

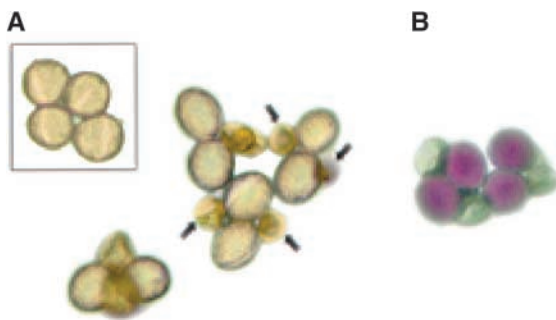


FIGURE 4.—Quartets of pollen grains from $+/aha3-5$, qrt/qrt plants. (A) Quartets containing two wild-type and two $aha3-5$ pollen grains. Arrows point to $aha3-5$ pollen grains (may be out of plane of focus). Inner box shows a quartet from a $+/+$, qrt/qrt plant. (B) Quartets containing two wild-type and two $aha3-5$ pollen grains, colored with Alexander’s viability stain.

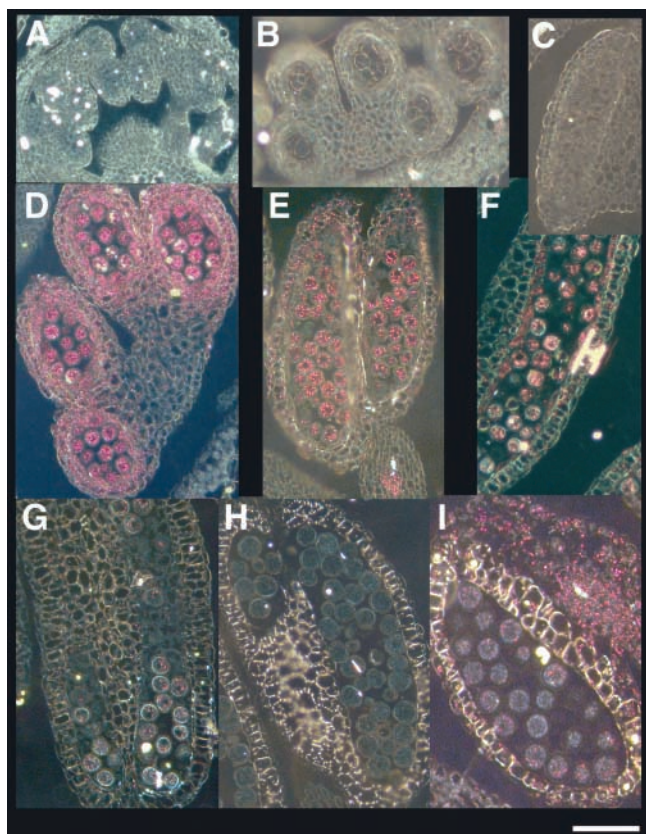


FIGURE 5.—Cross sections of an anther from a plant expressing *AHA3::GUS*, viewed through dark-field optics. (A) Pollen mother cells. (B) Tetrads. (C) Prevacuolate microspores. (D) Early vacuolated microspores. (E) Vacuolate microspores. (F) Late vacuolated microspores. (G) Mitotic microspores. (H) Mature pollen. (I) Cross section of an anther from a plant expressing *CPK28::GUS*. Bar, 30 μ M.

nificant because it confirmed that *AHA3* is indeed expressed during the early stage of microspore development at which lethality was incurred in *AHA3* knockout plants. It should be noted that our findings contradict our own earlier reports of *AHA3* expression in mature pollen.

It was possible that the GUS substrate was unable to penetrate the mature pollen grains, resulting in a perceived absence of *AHA3* expression in these tissues. To confirm that *AHA3* expression was truly absent in mature pollen, flowers containing a *CPK28::GUS* construct were sectioned and analyzed. *CPK28*, a gene encoding a member of the calcium-dependent protein kinase family, is expressed in various floral tissues, including mature pollen (C. W. CHAN, personal communication). Sections of *CPK28::GUS* flowers revealed GUS activity in mature pollen, seen as bright-pink crystals when viewed using dark-field optics (Figure 5I). This confirmed that GUS substrate was able to reach mature pollen and confirmed the absence of *AHA3* expression in these tissues.

Molecular complementation of *aha3-1*: Plants containing two copies of the *aha3-1* allele were not recoverable, presumably due to the lethal effect of the *aha3-1*

TABLE 4
Molecular complementation of *aha3-1*

T ₀ genotype	Kan ^S :Kan ^R	% T-DNA transmission	Rescued mutants
<i>AHA3/aha3-1</i>	93:151	61.9	
<i>AHA3/aha3-1</i>	98:184	65.2	
<i>AHA3/aha3-1</i>	60:80	57.1	
<i>AHA3/aha3-1</i>	91:122	57.3	5 (16)
<i>AHA3/aha3-1</i>	80:111	58.1	
<i>AHA3/aha3-1</i>	161:204	55.9	3 (16)
<i>AHA3/aha3-1</i>	91:115	55.8	
<i>AHA3/aha3-1</i>	70:95	57.6	6 (16)

T₀ refers to plants whose progeny were transformed with *pCAMBIA3300S::AHA3*. Kan^S:Kan^R refers to the ratio of sensitivity:resistance to 50 μ g/ml kanamycin observed among the progeny (T₂) of Kan^R transformants. Kan^R implies the presence of the *aha3-1* allele. % T-DNA transmission is the percentage of Kan^R of the total sampled. Rescued mutants were identified among the T₂ plants (number sampled).

mutation. To demonstrate causality, a full-length copy of *AHA3* was reintroduced, and *aha3-1/aha3-1* plants containing the transgene were identified among the transformants.

A full-length copy of *AHA3*, including 3 kb of upstream regulatory sequence and 500 bp of downstream regulatory sequence, was introduced into plants heterozygous for the *aha3-1* allele by *A. tumefaciens*-mediated transformation (Figure 1). We used a PCR-based strategy to identify rescued plants. This strategy utilized two pairs of primers—one pair specific to *AHA3* in its native, chromosomal location and a second pair specific to *aha3-1*. A rescued plant was defined as a plant from which PCR products specific to *aha3-1* could be amplified, but from which PCR products specific to native, wild-type *AHA3* could not be obtained.

The results of the complementation analysis are presented in Table 4. Analysis of Kan^R T₂ plants from eight independent lines revealed that the transmission of *aha3-1* increased following complementation with a wild-type copy of *AHA3*. Whereas transmission of *aha3-1* was originally 50% (through the female only), following complementation it was between 55 and 65%. This increase reflected transmission of *aha3-1* through both the female gamete, and, to some degree, the male gamete. Three lines were selected for further analysis. In each case, multiple plants homozygous for the *aha3-1* allele, yet alive due to the presence of the *AHA3* transgene, were identified.

Visualization of pollen grains stained with Alexander's stain was used to further confirm that the rescued plants were no longer affected by male gametophyte lethality due to the absence of a functional copy of *AHA3*. Pollen grains from a rescued *aha3-1/aha3-1* plant were stained and viewed as a uniform assortment of full, round, purple grains with green outlines (data not shown).

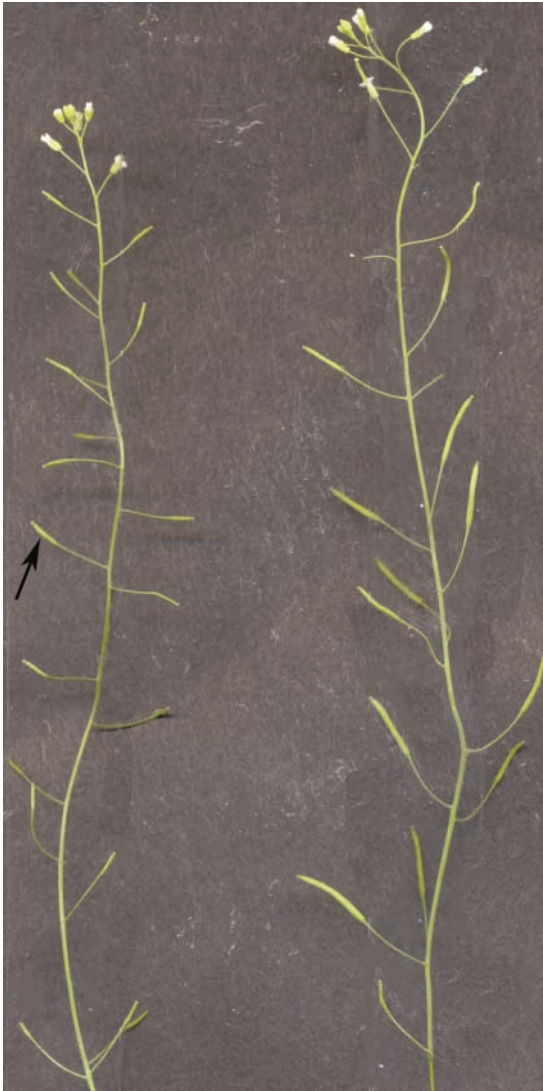


FIGURE 6.—Aerial tissues of a $+/aha3-5; AHA3T948A$ plant (left) and a $+/+; AHA3T948A$ plant. The $+/aha3-5; AHA3T948A$ plant is male sterile. Arrow points to an empty silique. Anthers from this plant lack pollen. The $+/+; AHA3T948A$ plant looks normal and is fertile.

Characterization of plants containing site-directed AHA3 mutants: To test the requirement for the penultimate threonine residue (T948), we replaced it with alanine (T948A) or aspartate (T948D) via site-directed mutagenesis. Constructs containing these mutations (*AHA3T948A*, *AHA3T948D*) were introduced into *AHA3/aha3-5* plants using the *Agrobacterium*-mediated floral dip method. Transformants (T_1) were selected and PCR genotyping was performed to determine the identity of each transformant. As expected, an approximately equal ratio of plants homozygous for the wild-type *AHA3* allele or heterozygous for *aha3-5* was recovered from each parent plant. However, the morphology of these plants differed depending on the genetic background and the specific transgene. While all $+/+; AHA3T948A$ plants appeared normal, three of five $+/aha3-5; AHA3T948A$ plants were completely male sterile (Figure 6). All siliques were empty,

and no pollen could be detected. Pistils fertilized with wild-type pollen filled out, indicating that the female organs were unaffected in these plants (data not shown). Due to the sterility of these plants, no progeny were obtained, and thus it was not possible to identify rescued mutants containing the *AHA3T948A* transgene (*aha3-5/aha3-5; AHA3T948A*). The remaining two of the five $+/aha3-5; AHA3T948A$ plants were normal in appearance, with fully developed siliques. Progeny from both of these lines were PCR analyzed, but no plants homozygous for *aha3-5* were identified.

The results were different for plants transformed with the *AHA3T948D* construct. In the T_1 generation, both $+/+; AHA3T948D$ plants and $+/aha3-5; AHA3T948D$ plants appeared normal and fertile. Progeny from $+/aha3-5; AHA3T948D$ plants were PCR analyzed, and plants homozygous for *aha3-5* were identified.

The growth of seedlings from one *aha3-5/aha3-5; AHA3T948D* line was tested on media at pH 4.5, 5, and 5.5. An earlier study indicated that removal of a C-terminal fragment of AHA3, including T948, conferred a growth advantage to seedlings on media at acidic pH (YOUNG *et al.* 1998). We analyzed *aha3-5/aha3-5; AHA3T948D* seedlings for similar growth characteristics. Hypocotyl length was measured as an indicator of growth. The results are presented in Table 5. The average hypocotyl length of both transgenic and wild-type seedlings increased with greater pH, but at each pH value, the average hypocotyl length of *AHA3T948D* seedlings was significantly greater than that of wild-type seedlings.

DISCUSSION

In this report, we used a reverse genetic strategy to explore the *in planta* requirement of a plasma membrane H^+ -ATPase. We also tested the requirement for a highly conserved threonine residue for pump activation. Our results suggest that expression of the H^+ -ATPase and a negative charge at the site of this threonine residue are essential.

Using different insertional mutant alleles, we demonstrated that eliminating the expression of a specific member of the Arabidopsis family of plasma membrane H^+ -ATPases was lethal. The presence of a T-DNA insertion in *AHA3* resulted in a 1:1 segregation of wild-type plants to heterozygotes from a heterozygous parent. This ratio is symptomatic of gametophytic lethality. Reciprocal crosses revealed that the source of the lethality was the male gamete. Null *aha3* alleles resulted in pollen abortion during the early vacuolated microspore stage. Because transmission of the mutant allele through the male gamete was impossible, no homozygous *aha3* mutant plants were recovered.

The timing of pollen abortion in the *aha3* plant most likely coincided with the onset of *AHA3* expression during the early vacuolated microspore stage of microgametogenesis. Our observation of *AHA3* expression in early stages of microgametogenesis is supported by microarray

TABLE 5
Growth of transgenic and wild-type seedlings on media at different pH

Seedling genotype	Average hypocotyl length (cm) ^a at pH:		
	4.5	5	5.5
<i>aha3-5/aha3-5; AHA3T948D</i>	0.95 ± 0.17	1.22 ± 0.22	1.37 ± 0.25
<i>AHA3/AHA3</i>	0.63 ± 0.11	0.74 ± 0.13	0.89 ± 0.16

^a 30 seedlings were tested per treatment.

analysis of transcripts from unicellular, bicellular, tricellular, and mature pollen (HONYS and TWELL 2003). The most likely role of *AHA3* in growing microspores is the control of nutrient uptake. As their dependence on the surrounding sporophytic tissues (tapetum) for nourishment decreases, early vacuolated microspores rely increasingly on the activity of the plasma membrane H⁺-ATPase for the transport of nutrients. If this protein is nonfunctional, the microspores starve and die. In agreement with this, the morphological contrast between the aborted mutant microspores and the full-sized, wild-type microspores is most obvious at the mature pollen stage.

The essentiality of a plasma membrane H⁺-ATPase was originally demonstrated in yeast. Yeast contain two genes encoding plasma membrane H⁺-ATPases, and knockouts of one of these genes are lethal (SERRANO *et al.* 1986). Of the 11 members of the Arabidopsis plasma membrane H⁺-ATPase family, T-DNA mutants have been reported previously for only one, *AHA4*. *AHA4* was shown to be localized to root endodermis and flowers, and growth of an *aha4* knockout was significantly reduced only when plants were grown in high salt (VITART *et al.* 2001). This suggested a role for this gene in resistance to salt stress. However, the phenotype was subtle and the mutants were not fully rescuable, indicating that the mutation was leaky and/or conferred semidominance. The *aha3* mutation described herein is the first among the *AHA* family members to have a clear and essential phenotype, and similar observations with other single *AHA* knockouts have not been seen (YOUNG *et al.* 2001).

On the basis of previous work describing *AHA3* localization in companion cells, we predicted a role for this gene in phloem loading, and consequently that an *AHA3* knockout plant would be deficient in this process. Along these lines, transgenic tobacco plants, in which the phloem-localized plasma membrane H⁺-ATPase is cosuppressed, are stunted and contain excess sugar in their leaves, among other phenotypes (ZHAO *et al.* 2000). Our results, which revealed male gametophyte lethality, were unexpected. The essentiality of *AHA3* in pollen precluded studies in which the role of *AHA3* in phloem function could be examined in a null background. For these experiments, it will be necessary to express wild-type copies of *AHA3* in mutant plants using a pollen-specific promoter or to identify sublethal alleles of *AHA3*.

Male gametophyte lethality in Arabidopsis can be

manifested a number of times during pollen development and function. For example, the male gametophyte mutants *mad2* and *mad3* are defective during pollen mitosis I, which occurs after reabsorption of the vacuoles in vacuolated microspores (GRINI *et al.* 1999); *mad1* is defective during pollen mitosis II (GRINI *et al.* 1999), as is *ttt38* (PROCISSI *et al.* 2001); *mad4* is defective in pollen tube elongation (GRINI *et al.* 1999); and *raring-to-go* causes precocious germination in the anther (JOHNSON and MCCORMICK 2001). An estimate of the frequency of male gametophytic lethality in a T-DNA-mutagenized population is 0.2% (PROCISSI *et al.* 2001), but in most cases the effect is not completely penetrant. In addition, female gametophyte lethality is also often observed. These cases therefore do not represent fully penetrant, male-specific gametophyte lethality. Only seven cases cite mutations that cause fully penetrant, male-specific gametophyte lethality; for only one of these mutations was the gene identified (*ADLIC*, a dynamin-like gene; KANG *et al.* 2003). *AHA3* is therefore only the second known gene for which fully penetrant, male gametophyte lethality is observed in the knockout.

We tested the requirement for a residue thought to play a critical role in H⁺-ATPase activation. According to the current model H⁺-ATPase activity under resting conditions is reduced because of an autoinhibitory effect applied by the C terminus (MORSOMME and BOUTRY 2000; PALMGREN 2001). The role of this domain was confirmed by several studies, which showed that truncation or displacement of the C terminus of plant H⁺-ATPases yielded a constitutively activated enzyme when expressed in yeast (PORTILLO and SERRANO 1989; PALMGREN *et al.* 1990; PORTILLO *et al.* 1991; PALMGREN and CHRISTENSEN 1993; REGENBERG *et al.* 1995). Activation *in vivo* is initiated by phosphorylation of the highly conserved penultimate threonine residue (T948 in *AHA3*). The phosphorylated threonine is recognized and bound by a 14-3-3 protein dimer (FUGLSANG *et al.* 1999; SVENNELID *et al.* 1999; MAUDOUX *et al.* 2000). This binding is thought to alter the orientation of the autoinhibitory C-terminal domain so that it is displaced from the active site of the ATPase, which relieves inhibition and activates the pump (OLSSON *et al.* 1998; FUGLSANG *et al.* 1999; CAMONI *et al.* 2000).

To test the role of T948 in *AHA3* regulation *in planta*, we replaced it with alanine or aspartate and characterized the effects of these mutant transgenes in plants with

a null *aha3* background. We predicted that substitution with alanine would produce a constitutively inactive enzyme, as it is not possible to phosphorylate alanine residues. Conversely, we predicted that the negatively charged aspartate would mimic a constitutively phosphorylated threonine residue, producing a constitutively activated, or hyperactive, enzyme.

Plants with a null *aha3* background but that contained the *AHA3T948A* transgene were never identified among the progeny of *+ / aha3-5; AHA3T948A* plants. We proposed that substitution of threonine with alanine at the penultimate location in *AHA3* prevents phosphorylation of the 14-3-3 binding target, binding of the 14-3-3 protein dimer, and activation of the H⁺-ATPase. We argue that the constitutive inactivity of the altered H⁺-ATPase produces a perceived functional absence of this enzyme. However, while two of the five *+ / aha3-5; AHA3T948A* plants identified were normal in appearance, the remaining three were male sterile, suggesting that the *AHA3T948A* transgene behaved in a dominant fashion. One possible explanation for the observed male sterility could be the presence of this transgene in the tapetal cells, which are known to be extremely sensitive to alterations in enzyme activity (WU and CHEUNG 2000). Metabolic perturbations in these cells, caused by unusual *AHA3T948A* functions, could inhibit nourishment of developing microspores, resulting in male sterility. In nonsterile *+ / aha3-5; AHA3T948A* plants, expression of the transgene may have been insufficient to cause sterility due to copy number or position effect. The use of a pollen-specific promoter to drive *AHA3T948A* expression in *aha3* plants would directly test the effect of the amino acid substitution and remove the effects of such confounding factors.

Plants with an *aha3* null background but containing *AHA3T948D* were identified by consecutive selection on kanamycin and BASTA, and their identities were confirmed by PCR genotyping. This result indicates that the conservation of negative charge at the penultimate threonine residue yielded a functional enzyme that could complement the lethal effect of *aha3*. However, identification of these plants at less than the expected Mendelian frequency of 25% from a heterozygous parent suggests that this transgene did not rescue with full penetrance, perhaps because our *AHA3* transgene requires additional flanking sequence and/or its native chromosomal locus for optimal expression.

Seedlings containing the *AHA3T948D* transgene appeared to have a significant growth advantage over wild-type seedlings on media at low pH. This result suggests that the substitution at T948 produced a hyperactive H⁺-ATPase that may drive increased removal of toxic protons from the cytoplasm of vascular cells of seedlings, "insensitivity" to the acidic media, and superior growth (YOUNG *et al.* 1998).

The role of the penultimate threonine residue of plant H⁺-ATPases was previously tested by expression of mutants in yeast lacking their own pump or in cell-

free extracts. Replacement of threonine with alanine in the tobacco plasma membrane H⁺-ATPase, PMA2, prevented complementation of yeast H⁺-ATPase mutants (SVENNELID *et al.* 1999; MAUDOUX *et al.* 2000). Surprisingly, replacement of threonine with aspartate also prevented complementation, but a negative result is difficult to interpret since effects of this mutation on biogenesis or turnover in this heterologous organism were not determined. In another study, replacement of threonine with alanine in *AHA2* prevented complementation of mutant yeast unless additional mutations in the C terminus were also present (JAHN *et al.* 2002). When the 14-3-3 protein-binding target was destroyed, but mutations in autoinhibitory regions in the C terminus were present, the mutant yeast was rescued. This demonstrated that activation of the H⁺-ATPase is not dependent on 14-3-3 protein binding *per se*, but on displacement or neutralization of the C terminus. Finally, substitution of alanine for the penultimate threonine of *AHA2* prevented phosphorylation at this site, 14-3-3 binding, and ATP hydrolysis *in vitro*, further illustrating the requirement for threonine in pump function (FUGLSANG *et al.* 1999).

Our results help define the role of the penultimate threonine residue in plasma membrane H⁺-ATPase activity *in planta*. First, we showed that substitution of the penultimate threonine residue with alanine prevents complementation of the mutant plant. This finding is in agreement with results from the heterologous experiments described above. Second, we showed that replacement of the penultimate threonine with aspartate produces an active H⁺-ATPase that is capable of rescuing transmission of *aha3* through the male gamete.

Through segregation analyses, microscopic analysis of mutant reproductive tissues, confirmation of *AHA3* expression in the appropriate microspore stages, complementation of the male gametophyte lethality with a wild-type copy of *AHA3*, and confirmation of gametophytic lethality by crossing to *qrt*, we have identified an essential function for *AHA3* in microspore development. We suggest that this role involves control of nutrient acquisition, although other functions, such as in cell-wall deposition or intercellular signaling, cannot be ruled out. We have also confirmed the requirement for a negatively charged amino acid at the penultimate location in the regulatory C terminus.

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LITERATURE CITED

- ALEXANDER, M. P., 1969 Differential staining of aborted and non-aborted pollen. *Stain Technol.* **44**: 117-122.

- CAMONI, L., V. IORI, M. MARRA and P. ADUCCI, 2000 Phosphorylation-dependent interaction between plant plasma membrane H⁺-ATPase and 14-3-3 proteins. *J. Biol. Chem.* **275**: 9919–9923.
- CLOUGH, S. J., and A. F. BENT, 1998 Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**: 735–743.
- DEWITT, N. D., and M. R. SUSSMAN, 1995 Immunocytological localization of an epitope-tagged plasma membrane proton pump (H⁺-ATPase) in phloem companion cells. *Plant Cell* **7**: 2053–2067.
- DEWITT, N. D., J. F. HARPER and M. R. SUSSMAN, 1991 Evidence for a plasma membrane proton pump in phloem cells of higher plants. *Plant J.* **1**: 121–128.
- FERNANDEZ, D. E., G. R. HECK, S. E. PERRY, S. E. PATTERSON, A. B. BLEECKER *et al.*, 2000 The embryo MADS domain factor AGL15 acts postembryonically. Inhibition of perianth senescence and abscission via constitutive expression. *Plant Cell* **12**: 183–198.
- FUGLSANG, A. T., S. VISCONTI, K. DRUMM, T. JAHN, A. STENSALLE *et al.*, 1999 Binding of 14-3-3 protein to the plasma membrane H⁺-ATPase AHA2 involves the three C-terminal residues Tyr(946)-Thr-Val and requires phosphorylation of Thr(947). *J. Biol. Chem.* **274**: 36774–36780.
- GRINI, P. E., A. SCHNITTGER, H. SCHWARZ, I. ZIMMERMANN, B. SCHWAB *et al.*, 1999 Isolation of ethyl methanesulfonate-induced gametophytic mutants in *Arabidopsis thaliana* by a segregation distortion assay using the multimarker chromosome 1. *Genetics* **151**: 849–863.
- HARPER, J. F., L. MANNEY, N. D. DEWITT, M. H. YOO and M. R. SUSSMAN, 1990 The *Arabidopsis thaliana* plasma membrane H⁺-ATPase multigene family. Genomic sequence and expression of a third isoform. *J. Biol. Chem.* **265**: 13601–13608.
- HIRSCH, R. E., B. D. LEWIS, E. P. SPALDING and M. R. SUSSMAN, 1998 A role for the AKT1 potassium channel in plant nutrition. *Science* **280**: 918–921.
- HONYS, D., and D. TWELL, 2003 Comparative analysis of the *Arabidopsis* pollen transcriptome. *Plant Physiol.* **132**: 640–652.
- HOULNE, G., and M. BOUTRY, 1994 Identification of an *Arabidopsis thaliana* gene encoding a plasma membrane H⁺-ATPase whose expression is restricted to anther tissue. *Plant J.* **5**: 311–317.
- JAHN, T. P., A. SCHULZ, J. TAIPALENSUU and M. G. PALMGREN, 2002 Post-translational modification of plant plasma membrane H⁺-ATPase as a requirement for functional complementation of a yeast transport mutant. *J. Biol. Chem.* **277**: 6353–6358.
- JOHNSON, S. A., and S. McCORMICK, 2001 Pollen germinates precociously in the anthers of raring-to-go, an *Arabidopsis* gametophytic mutant. *Plant Physiol.* **126**: 685–695.
- KANG, B. H., D. M. RANCOUR and S. Y. BEDNAREK, 2003 The dynamine-like protein ADL1C is essential for plasma membrane maintenance during pollen maturation. *Plant J.* **35**: 1–15.
- KOPREK, T., S. RANGEL, D. McELROY, J. D. LOUWERSE, R. E. WILLIAMS-CARRIER *et al.*, 2001 Transposon-mediated single-copy gene delivery leads to increased transgene expression stability in barley. *Plant Physiol.* **125**: 1354–1362.
- KRYSAN, P. J., J. C. YOUNG, F. TAX and M. R. SUSSMAN, 1996 Identification of transferred DNA insertions within *Arabidopsis* genes involved in signal transduction and ion transport. *Proc. Natl. Acad. Sci. USA* **93**: 8145–8150.
- KRYSAN, P. J., J. C. YOUNG and M. R. SUSSMAN, 1999 T-DNA as an insertional mutagen in *Arabidopsis*. *Plant Cell* **11**: 2283–2290.
- MATZKE, A. J., and M. A. MATZKE, 1998 Position effects and epigenetic silencing of plant transgenes. *Curr. Opin. Plant Biol.* **1**: 142–148.
- MAUDOUX, O., H. BATOKO, C. OECKING, K. GEVAERT, J. VANDEKERCKHOVE *et al.*, 2000 A plant plasma membrane H⁺-ATPase expressed in yeast is activated by phosphorylation at its penultimate residue and binding of 14-3-3 regulatory proteins in the absence of fusicoccin. *J. Biol. Chem.* **275**: 17762–17770.
- MORSOMME, P., and M. BOUTRY, 2000 The plant plasma membrane H⁺-ATPase: structure, function and regulation. *Biochim. Biophys. Acta* **1465**: 1–16.
- MOULINE, K., A. A. VERY, F. GAYMARD, J. BOUCHEREZ, G. PILOT *et al.*, 2002 Pollen tube development and competitive ability are impaired by disruption of a Shaker K⁺ channel in *Arabidopsis*. *Genes Dev.* **16**: 339–350.
- OLSSON, A., F. SVENNELID, B. EK, M. SOMMARIN and C. LARSSON, 1998 A phosphothreonine residue at the C-terminal end of the plasma membrane H⁺-ATPase is protected by fusicoccin-induced 14-3-3 binding. *Plant Physiol.* **118**: 551–555.
- PALMGREN, M. G., 2001 Plant plasma membrane H⁺-ATPases: powerhouses for nutrient uptake. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52**: 817–845.
- PALMGREN, M. G., and G. CHRISTENSEN, 1993 Complementation *in situ* of the yeast plasma membrane H⁺-ATPase gene PMA1 by an H⁺-ATPase gene from a heterologous species. *FEBS Lett.* **317**: 216–222.
- PALMGREN, M. G., C. LARSSON and M. SOMMARIN, 1990 Proteolytic activation of the plant plasma membrane H⁺-ATPase by removal of a terminal segment. *J. Biol. Chem.* **265**: 13423–13426.
- PORTILLO, F., and R. SERRANO, 1989 Growth control strength and active site of yeast plasma membrane ATPase studied by site-directed mutagenesis. *Eur. J. Biochem.* **186**: 501–507.
- PORTILLO, F., P. ERASO and R. SERRANO, 1991 Analysis of the regulatory domain of yeast plasma membrane H⁺-ATPase by directed mutagenesis and intragenic suppression. *FEBS Lett.* **287**: 71–74.
- PREUSS, D., S. Y. RHEE and R. W. DAVIS, 1994 Tetrad analysis possible in *Arabidopsis* with mutation of the QUARTET (QRT) genes. *Science* **264**: 1458–1460.
- PROCISSI, A., S. DE LAISSARDIERE, M. FERAULT, D. VEZON, G. PELLETIER *et al.*, 2001 Five gametophytic mutations affecting pollen development and pollen tube growth in *Arabidopsis thaliana*. *Genetics* **158**: 1773–1783.
- REGAN, S. M., and B. A. MOFFATT, 1990 Cytochemical analysis of pollen development in wild-type *Arabidopsis* and a male-sterile mutant. *Plant Cell* **2**: 877–889.
- REGENBERG, B., J. M. VILLALBA, F. C. LANFERMEIJER and M. G. PALMGREN, 1995 C-terminal deletion analysis of plant plasma membrane H⁺-ATPase: yeast as a model system for solute transport across the plant plasma membrane. *Plant Cell* **7**: 1655–1666.
- SERRANO, R., M. C. KIELLAND-BRANDT and G. R. FINCK, 1986 Yeast plasma membrane ATPase is essential for growth and has homology with Na⁺/K⁺, K⁺- and Ca²⁺-ATPases. *Nature* **319**: 689–693.
- SVENNELID, F., A. OLSSON, M. PIOTROWSKI, M. ROSENQUIST, C. OTTMAN *et al.*, 1999 Phosphorylation of Thr-948 at the C terminus of the plasma membrane H⁺-ATPase creates a binding site for the regulatory 14-3-3 protein. *Plant Cell* **11**: 2379–2391.
- VITART, V., I. BAXTER, P. DOERNER and J. F. HARPER, 2001 Evidence for a role in growth and salt resistance of a plasma membrane H⁺-ATPase in the root endodermis. *Plant J.* **27**: 191–201.
- WU, H., and A. Y. CHEUNG, 2000 Programmed cell death in plant reproduction. *Plant Mol. Biol.* **44**: 267–281.
- YOUNG, J. C., N. D. DEWITT and M. R. SUSSMAN, 1998 A transgene encoding a plasma membrane H⁺-ATPase that confers acid resistance in *Arabidopsis thaliana* seedlings. *Genetics* **149**: 501–507.
- YOUNG, J. C., P. J. KRYSAN and M. R. SUSSMAN, 2001 Efficient screening of *Arabidopsis* T-DNA insertion lines using degenerate primers. *Plant Physiol.* **125**: 513–518.
- ZHAO, R., V. DIELEN, J. M. KINET and M. BOUTRY, 2000 Cosuppression of a plasma membrane H⁺-ATPase isoform impairs sucrose translocation, stomatal opening, plant growth, and male fertility. *Plant Cell* **12**: 535–546.

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