Corrections

GENETICS. For the article “A non-ATP-competitive inhibitor of BCR-ABL overrides imatinib resistance,” by Kiranmai Gumireddy, Stacey J. Baker, Stephen C. Cosenza, Premila John, Anthony D. Kang, Kimberly A. Robell, M. V. Ramana Reddy, and E. Premkumar Reddy, which appeared in issue 6, February 8, 2005, of Proc. Natl. Acad. Sci. USA (102, 1992–1997; first published January 27, 2005; 10.1073/pnas.0408283102), the authors note that an incorrect image was published as Fig. 1B. The correct figure and its legend appear below. This correction does not affect the conclusions of the article.

**Fig. 1.** BCR-ABL inhibitory activity of ON012380. (A) Structure of ON012380. (B and C) Ten nanograms of recombinant BCR-ABL protein was mixed with different concentrations of the indicated inhibitor, and kinase assays were performed by using Crk as a substrate to measure autophosphorylation and substrate (Crk) phosphorylation. (D) BCR-ABL kinase assays were performed as described in Materials and Methods by using c-Crk as a substrate. The reactions mixtures were spotted onto strips of P81 phosphocellulose paper, washed, and counted. In experiments for which a mixture of imatinib and ON012380 was used, the reaction mixtures contained a constant amount of imatinib (10 nM) and various amounts of ON012380. The values from individual samples were analyzed and plotted as a function of drug concentration. Data points represent an average of three independent experiments performed in duplicate.

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PLANT BIOLOGY. For the article “A plasma membrane H⁺-ATPase is required for the formation of proanthocyanidins in the seed coat endothelium of Arabidopsis thaliana,” by Ivan R. Baxter, Jeffery C. Young, Gordon Armstrong, Nathan Foster, Naomi Bogenschutz, Tatiana Cordova, Wendy Ann Peer, Samuel P. Hazen, Angus S. Murphy, and Jeffrey F. Harper, which appeared in issue 7, February 15, 2005, of Proc. Natl. Acad. Sci. USA (102, 2649–2654; first published February 4, 2005; 10.1073/pnas.0406377102), due to a printer’s error, Ivan R. Baxter should have been credited in all categories of author contributions, and Jeffery C. Young should have been credited for writing the paper. The corrected author contributions footnote, which appears online only, is below.


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Department of Medical Pharmacology, Consiglio Nazionale delle Ricerche, Institute of Neuroscience, Cellular and Molecular Pharmacology, Center of Excellence on Neurodegenerative Diseases, University of Milan, 20129 Milan, Italy

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A plasma membrane H\textsuperscript+-ATPase is required for the formation of proanthocyanidins in the seed coat endothelium of Arabidopsis thaliana

Ivan R. Baxter\textsuperscript{*†‡}, Jeffery C. Young\textsuperscript*§, Gordon Armstrong\textsuperscript*, Nathan Foster\textsuperscript*, Naomi Bogenschutz\textsuperscript*, Tatiana Cordova\textsuperscript*, Wendy Ann Peer\textsuperscript*, Samuel P. Hazen\textsuperscript*, Angus S. Murphy\textsuperscript*, and Jeffrey F. Harper\textsuperscript{***††}

\textsuperscript{*}Center for Phytoremediation Research and Development, \textsuperscript{§}Purdue–UT-Pan American Phytoremediation Program, and \textsuperscript{**}Department of Horticulture, Purdue University, West Lafayette, IN 47907; \textsuperscript{¶}Department of Biology, Western Washington University, Bellingham, WA 98225; \textsuperscript{***}Biochemistry Department, University of Nevada, MS200, Reno, NV 89557; and \textsuperscript{††}The Scripps Research Institute, La Jolla, CA 92037

Edited by Maarten Koornneef, Max Planck Institute for Plant Breeding Research, Cologne, Germany, and approved December 16, 2004 (received for review August 31, 2004)

The plasma membrane in plant cells is energized with an electrical potential and proton gradient generated through the action of H\textsuperscript{+} pumps belonging to the P-type ATPase superfamily. The Arabidopsis genome encodes 11 plasma membrane H\textsuperscript{+} pumps. Autoinhibited H\textsuperscript{+}-ATPase isoform 10 (AHA10) is expressed primarily in developing seeds. Here we show that four independent gene disruptions of AHA10 result in seed coats with a transparent testa phenotype (light-colored seeds). A quantitative analysis of extractable flavonoids in aha10 seeds revealed an \sim100-fold reductio

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Abbreviations: AHA10, autoinhibited H\textsuperscript{+}-ATPase isoform 10; PA, proanthocyanidin; MATE, multidrug and toxic compound extrusion; Ws, Wassilewskija; DMCAC, dimethylallyladnin; BAN, banyuls.

1I.R.B. and J.C.Y. contributed equally to this work.

To whom correspondence should be addressed. E-mail: jfharper@unr.edu.

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borders. The border sequences of genes, numbering from ATG) were determined by sequencing of PCR-amplified products. The results were verified by cloning and sequencing the isolated PCR products. Two additional lines in the Columbia (Col) background (aha10-4, SALK.019569 and aha10-5, SALK.048598.53.35) were obtained from the SIGnAL collection (18) and verified by PCR analysis with the left border primer. Seed for a tt12 mutant (Ws ecotype) was obtained from the Arabidopsis Biological Resource Center (CS5740). Plants were grown in soil under 24-h light conditions in a growth room at 20°C. All experiments were conducted on wild-type and mutant siliques or on seeds from plants that were grown side-by-side in the same soil container.

Complementation. The AHA10 gene was cloned as an Xhol/SpeI fragment from a genomic cosmid clone pCAK1 (4) into a pBin vector (PS249) encoding a glucosamine-aminomann estanol (BASTA)-resistance gene for plant expression (19). Mutant aha10-1 plants were transformed by using an Agrobacterium-mediated floral dip infiltration method (20). The T1 transgenic plants were selected on 0.5X Murashige and Skoog medium containing 10 μg/ml BASTA.

p-Dimethylaminocinnamaldehyde (DMACA) Assay. Seeds were incubated in 2% (wt/vol) DMACA in 3 M HCl/50% (vol/vol) methanol for 4 days (10). The seeds were then washed four times in 70% ethanol and visualized with a light microscope.

Flavonoid Quantitation. Nonanthocyanin flavonoids were extracted from seeds and analyzed as described in refs. 9 and 10, except that HPLC fractions were separated and analyzed by using a Supelco C18 column on a Waters Alliance 2900S HPLC system with diode array (DAD) monitoring of 200–600 nm. Briefly, the seeds were pulverized and extracted with acetone, Folch-partitioned, and extracted with hexane. For the quantitation of PA, extracts were further subjected to acid hydrolysis in the presence of phloroglucinol (J. T. Baker). Epicatechin analysis was conducted before phloroglucinol hydrolysis, because this hydrolysis releases the terminal epicatechin of the PA polymer. Authentic quercetin, kaempferol, and catechin standards were obtained from Sigma. Cyanidin and pelargonidin were a gift from Dr. Ralph Nicholson (Purdue University), and epicatechin was extracted from black tea (21). Cyanidin-based standards procyanidin B2 and B3 and pelargonidin-based standard pelargonidin chloride for phloroglucinol analysis were obtained from standard collections of Dr. Ralph Nicholson or from ChromaDex (Santa Ana, CA). We did not observe any peaks from Arabidopsis PA corresponding to catechin- or pelargonidin-derived adducts. Although the extraction of flavonoids from mature seeds was not complete, as indicated by residual pigment that was recalcitrant to solubilization, the ratios of flavonoids extracted in mature and developing seeds were the same (data not shown). The residual pigment was not extractable with isopropanol, chloroform/methanol/water 1:1:1, or ethyl acetate.

The anthocyanins were extracted from seeds by grinding in methanolic acid and were allowed to sit overnight at 4°C in the dark, Folch-partitioned (22), and analyzed by HPLC as described above.

Visualizing Vacuoles. Vacuoles were observed by using the fluorescent dye of carboxy 2',7'-dichlorofluorescin diacetate (Molecular Probes). Seeds were collected from mature green siliques in which embryos had developed past the torpedo stage (i.e., early mature stage). Seeds were dissected and prepared as described in ref. 9, and images were observed on a Type DM/LE microscope (Leica, Deerfield, IL). A Nikon E5000 digital camera was used to capture images.

Quantitative RT-PCR. Total RNA was extracted from the first 12 developing siliques closest to the apical meristem by using RNeasy kits (Qiagen, Valencia, CA). First-strand cDNA was synthesized by using a poly(dT) primer and Moloney murine leukemia virus reverse transcriptase (Promega) according to the manufacturer’s protocol. Taqman assays (Applied Biosystems) were performed on

Materials and Methods

Plant Lines and Growth Conditions. Plant lines containing T-DNA insertions in AtAHA10 (At1g17260) in the Wassilewskija (Ws) background were identified from the Wisconsin collection by PCR screening (aha10-1 and aha10-2) and were confirmed by sequencing (17). Two additional lines in the Columbia (Col) background (aha10-4, SALK.019569 and aha10-5, SALK.048598.53.35) were obtained from the SIGnAL collection (18) and verified by PCR analysis with the left border primer. Seed for a tt12 mutant (Ws ecotype) was obtained from the Arabidopsis Biological Resource Center (CS5740). Plants were grown in soil under 24-h light conditions in a growth room at 20°C. All experiments were conducted on wild-type and mutant siliques or on seeds from plants that were grown side-by-side in the same soil container.

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Results

AHA10 Gene Disruptions. As part of our effort to determine the in planta functions of all 11 Arabidopsis AHA s, we identified four independent T-DNA insertion plant lines harboring gene disruptions of AHA10. Two lines were obtained in the Ws ecotype from the Wisconsin collection (aha10-1 and aha10-2) (17) and two in the Col ecotype from the Salk collection (aha10-4 and aha10-5) (18) (Fig. 1b). Homozygous mutant plants were identified for all four mutations, indicating that the gene disruptions were not lethal. The chromosomal location of this aha10/tt mutation did not correspond to any well characterized tt mutant (23). PCR genotyping of segregating aha10-2 populations revealed normal Mendelian segregation of the aha10 mutation ($\chi^2 = 0.644, P = 0.7247, n = 118$). Extensive phenotypic analysis was restricted to the aha10-1 and aha10-2 alleles in the Ws background, although both Col background mutants showed a similar tt phenotype, as described below. The T-DNA insertion site in aha10-2 was sequenced and shown to be located in a coding sequence exon. The other three aha10 insertions were located in introns, as determined by border sequences.

Seeds from aha10 Plants Have a tt Phenotype. To assay for phenotypes in aha10 mutant lines, we conducted a variety of assays for plant growth and development under different growth conditions (see the supporting information, which is published on the PNAS web site). The only consistent difference observed was in seed coat color. Seeds from all four homozygous T-DNA insertion lines had pale seed coats (tt phenotype) under all growth conditions tested (Fig. 2a and b). This phenotype was not observed in hetrozygotes, indicating that all four gene disruptions represent recessive mutations.

The AHA10 Gene Complements. To confirm that the tt phenotype resulted from a loss of AHA10 function, we transformed aha10-1 plants with a genomic clone containing the AHA10 gene with 1.45 kb of upstream sequence and 1 kb of downstream sequence. Seeds from all 11 independent transgenic plant lines had a restored wild-type phenotype, as seen by a visual inspection of seed color as well as by staining for PA using DMACA (data not shown). Because our genomic clone did not contain any other predicted genes, complementation provides strong corroboration that AHA10 itself is required for normal pigmentation of Arabidopsis seed coats.

aha10 Mutants Have Normal Anthocyanin Levels but Reduced PA. The first tt mutants in Arabidopsis defined many of the key enzymes and proteins in flavonoid biosynthesis (11, 12, 24) (Fig. 3). Although the flavonoid pathway produces a wide variety of compounds, colored pigments in the Arabidopsis seed coat result primarily from the accumulation of anthocyanins and PA (8). The biosynthesis of both of these end-product pigments includes many shared upstream intermediates but diverges into two specific pathways after the production of cyanidin (Fig. 3).

To determine whether the PA and anthocyanin branches of the pathway were both affected in aha10 mutants, we quantified extractable levels of anthocyanin and PA in mature seeds. Although wild type and mutants showed comparable levels of anthocyanin, the mutants showed a >100-fold decrease in PA (Table 1). This significant decrease in PA ($P < 0.05$) was confirmed by staining seeds with DMACA (Fig. 2 c and d), which stains PA much more strongly than it stains other flavonoids. As expected, wild-type seeds stained black, indicating the presence of PA, whereas the aha10 seeds showed no detectable staining. Thus, aha10 seeds have a defect in flavonoid production that affects the PA pathway but not the anthocyanin pathway.

A Disruption in PA Production Occurs Downstream of Epicatechin. To delineate the point at which PA production is disrupted in the aha10 mutants, we analyzed the flavonoid profiles of mature seed extracts by using HPLC. Of the seven extractable flavonoids that were
quantified (Table 1), the end-product PA was the only flavonoid to consistently show a dramatic reduction ($P < 0.05$). In contrast, no significant changes were observed in anthocyanin (discussed above), kaempferol, or pelargonidin ($P > 0.05$). The colorless flavonol quercetin increased slightly ($P < 0.05$). These results provide strong evidence that the primary defect in aha10 mutants is specific to the PA biosynthetic branch.

In agreement with Abrahams et al. (9), we detected only epicatechin from our PA hydrolysis, indicating that Arabidopsis PA is composed exclusively of epicatechin monomers. This composition makes cyanidin the intermediate at the branch point that feeds into both the anthocyanin and PA pathways. Because anthocyanin was found at wild-type levels in the mutant, we hypothesized that the levels of the precursor cyanidin would not be reduced in the aha10 mutant. This expectation was verified, with cyanidin levels actually showing a slight increase (2.7-fold, $P < 0.05$).

In wild type, cyanidin is directly converted to epicatechin (the monomer unit of PA) in the first committed step of the PA pathway (25). This step is catalyzed by the enzyme BAN. To evaluate whether this PA-specific step was normal, we quantified soluble epicatechin directly from seed extracts. No free epicatechin was detectable in wild type, consistent with this intermediate occurring at very low levels. In contrast, significant levels of free epicatechin were detected in the mutants. Thus, the aha10 defect appears to cause a blockage resulting in the accumulation of epicatechin.

Vacuolar Biogenesis Is Altered in aha10 and tt12 Seed Coat Endothelial Cells. PA accumulates in the central vacuole of seed coat endothelial cells (5). To determine whether the loss of AHA10 affects the formation of these vacuoles, we performed microscopy with 5- (and -6-) carboxy 2',7'-dichlorofluorescein diacetate (Fig. 2 e and f), a dye previously used to visualize plant vacuoles (9). When developing seeds (in which embryos are in the early mature stage) were examined, a large central vacuole was observed in the wild-type seed coat endothelial cells. In contrast, the equivalent staged cells in aha10 seeds showed multiple smaller vacuoles. The endothelial cells in Fig. 2 are representative of images most commonly observed for both wild type and mutant. Although small vacuoles were observed in 96% of the mutant endothelial cells ($n = 77$), only 11% of cells from wild-type controls showed comparable morphologies ($n = 64$). As the seeds matured, the differences between wild type and mutant diminished, indicating that the mutation resulted in a developmental delay, as opposed to a complete block in vacuolar biogenesis. We also observed this phenotype in the developing seeds of the tt12 mutant, where 94% of the cells observed had small vacuoles ($n = 383$).

aha10 Does Not Reduce the Accumulation of BAN and TT12 mRNA. To test for an aha10-related deficiency in general functions of seed coat endothelial cells, we conducted quantitative RT-PCR for two mRNAs (BAN and TT12). Both BAN and TT12 are specifically expressed in seed coat endothelial cells and function in the PA-specific branch of flavonoid biogenesis. BAN catalyzes the first committed step in PA synthesis (Fig. 3) (25), whereas TT12 encodes

![Flavonoid pathway in Arabidopsis](Image)

**Fig. 3.** Flavonoid pathway in Arabidopsis. Pathway diagram is derived from ref. 9. The known elements of the flavonoid pathway are indicated. The protein names are capitalized; the corresponding mutant line in Arabidopsis is in italics. The different chemical species are indicated in lowercase. Bold letters indicate flavonoids quantified in Table 1. End products are boxed. Dotted line perpendicular to arrow indicates a hypothetical membrane transport step. Plants with disruptions in transcription factor genes TT1, TT2, TT3, TT6, TTG1, and TTG2 have been shown to have tt phenotypes, as well. tds, tannin deficient seed; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavone 3 hydroxylase; F3′H, flavone 3′ hydroxylase; DFR, dihydroflavonol reductase; F3′H, flavone 3′ hydroxylase; UFGT, UDP-glucose-flavonoid 3-O-glucosyl transferase.

### Table 1. Quantitation of flavonoids in mature seeds shows mutants with decreased PA

<table>
<thead>
<tr>
<th>Flavonoids</th>
<th>Ws</th>
<th>aha10-1</th>
<th>aha10-2</th>
<th>Change from wild type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaempferol</td>
<td>0.3 (64)</td>
<td>0.076 (5)</td>
<td>0.10 (21)</td>
<td>Not significant</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.216 (0.07)</td>
<td>0.40 (8)</td>
<td>1.22 (3)</td>
<td>Up $\geq$1.8-fold</td>
</tr>
<tr>
<td>Pelargonidin</td>
<td>0.001 (35)</td>
<td>0.01 (312)</td>
<td>0.001 (137)</td>
<td>Not significant</td>
</tr>
<tr>
<td>Cyanidin</td>
<td>0.05 (26)</td>
<td>0.14 (12)</td>
<td>0.27 (23)</td>
<td>Up $\geq$2.7-fold</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>Pre-PG</td>
<td>0*</td>
<td>0.08 (55)</td>
<td>0.028 (81)</td>
</tr>
<tr>
<td>PA</td>
<td>12.5 (8)</td>
<td>0.18 (103)</td>
<td>0.10 (47)</td>
<td>Down $\geq$100-fold</td>
</tr>
<tr>
<td>Anthocyanin</td>
<td>7.8 (27)</td>
<td>5.8 (26)</td>
<td>6.2 (9)</td>
<td>Not significant</td>
</tr>
</tbody>
</table>

*No epicatechin was detected in the Ws seed extracts before PG treatment.

The levels of each flavonoid are presented with the percent standard deviation in parentheses. Significance determined by Student’s $t$ test, $P < 0.05$, $n = 3$. PG, phloroglucinol treatment; PA EU, PA extension units (epicatechin-phloroglucinol). All values are in μg of flavonoid/g of seed except anthocyanins, which are expressed as nmol/g of seed.

**Table 1. Quantitation of flavonoids in mature seeds shows mutants with decreased PA**
a MATE transporter that potentially functions to transport epicatechin into vacuoles (13). To evaluate the relative expression levels of these two endothelial cell-specific transcripts in mutants and wild type, we compared their expression values with two mRNA trascripts that are expressed in many cell types of the plant (IPP2 and APX3).

The RNA template used for RT-PCR was extracted from the uppermost 12 green siliques located closest to the apical meristem (Fig. 4). In each of three independent experiments, the relative expression levels of BAN and TT12 were slightly higher in the mutant than in the wild type. This finding shows that the expression of these two PA pathway genes is not the rate-limiting step in PA biosynthesis in an aha10 mutant. More generally, it provides further evidence that the aha10 disruption does not compromise essential cellular functions (e.g., the plasma membrane electrical potential and proton motive force) that would result in a global decrease in gene expression.

Discussion

**aha10 Disrupts the Biosynthesis of PA.** Evidence presented here shows that four independent homozygous gene disruptions of a plasma membrane proton pump AHA10 (Fig. 1) all result in a tt phenotype (Fig. 2). These mutants appear to result from a loss of AHA10 function, as indicated by the ability to complement aha10-1 by using an AHA10 genomic clone. The underlying cause of this tt phenotype appears to be related to a 100-fold reduction in PA (Table 1). PA is produced in seed coat endothelial cells and accumulates in large central vacuoles. Because AHA10 is classified as a plasma membrane proton pump, the aha10/tt phenotype provides an unexpected connection between the function of a putative plasma membrane proton pump and the accumulation of a specific flavonoid in the central vacuole.

Although the exact composition of the colored pigments in the *Arabidopsis* seed coat is unknown, it has been attributed to anthocyanin and PA (8, 13). A complete loss of anthocyanidins and anthocyanins results in a yellow seed, as observed in a chalcone synthase-deficient mutant that produces no flavonoids (24) and the dihydroflavonol 4-reductase mutants that only produce flavonols (26). In contrast, the aha10 mutants show only a slight reduction in seed color. PA is the only major end-product flavonoid that is missing in aha10 mutants, suggesting that it is not PA, but anthocyanins or an unextracted pigment, that accounts for the major contribution to seed coat color in *Arabidopsis*.

The aha10 mutations do not completely eliminate any flavonoid biosynthetic branch in seeds, as evidenced by the detection of all three primary end products of the flavonoid biosynthetic pathway (PA, anthocyanin, and flavonols). Nevertheless, aha10 dramatically and specifically affects the PA-biosynthetic branch. This phenotype was revealed by the quantitation of seven different extractable seed flavonoids (Table 1). In this analysis, aha10 mutants showed either no significant change or increased levels of the end products anthocyanin and the flavonols kaempferol and quercetin, in contrast to a 100-fold reduction in the end product PA. Elevated concentrations were also observed for cyanidin, an intermediate that lies at the fork in the branched pathway leading to either PA or anthocyanin. This finding indicates that the defect in aha10 occurs at a step downstream of cyanidin biosynthesis.

In the downstream conversion of cyanidin to PA, epicatechin is the only known chemical intermediate. The biosynthetic steps leading to epicatechin are posited to occur in the cytosol, although the enzymatic machinery is membrane-attached (27–29). Epicatechin is thought to be the intermediate that is transported into vacuoles (5). Once transported into a vacuole, condensation of epicatechin into PA may occur nonenzymatically in response to an acidic environment (30). In wild type, we did not observe any unpolymerized epicatechin, consistent with it being a low-abundance and transient intermediate. In contrast, we detected significant levels of unpolymerized epicatechin in mutants. This increase in epicatechin is consistent with a pathway “backup” resulting from a block in epicatechin transport into vacuoles or its conversion into PA.

**aha10 and tt12 Disrupt Vacular Biogenesis.** In different plant cells, there can be multiple vacuoles with distinct functions (31, 32). Some plant vacuoles may originate directly from the endoplasmic reticulum; others may require contributions from the Golgi or plasma membrane. Very little is known about the biogenesis of the specialized vacuoles in seed coat endothelial cells (15).

In aha10 mutants, we observed a predominance of small vacuoles, as opposed to one large central vacuole, in young developing seed coat endothelial cells (Fig. 2). This phenotype appears similar to those observed for gene disruptions of *LDOX* (lueoanthocyanidin dioxygenase) (9) and TT12-MATE (shown in this study). LDOX is an oxygenase that converts leucocyanidin to cyanidin. In the *ldox* mutant, the production of downstream flavonoids, such as epicatechin and PA, are blocked, and the endothelial cells develop with an abundance of small vacuoles. Although it is not clear how the *ldox* mutation prevents normal vacuolar biogenesis, two general mechanisms have been proposed. First, the *ldox* mutation may result in the buildup of a toxic upstream intermediate that somehow disrupts vacuolar biogenesis. Alternatively, it may block the production of a downstream intermediate that is required as a positive metabolic trigger for provacuolar maturation. Although the mechanism by which *aha10* and *tt12* disrupt vacuolar biogenesis is also unclear, all three mutations could cause a buildup in the same toxic intermediate or block the synthesis or accumulation of a positive metabolic trigger.

**A Model for AHA10’s Function in Flavonoid and Vacular Biogenesis.** Although the expected function of a plasma membrane proton pump is to energize the plasma membrane and thereby enable signaling and solute transport at the cell surface, these established functions do not readily explain the specific vacuolar and PA biosynthesis defects observed for aha10 mutants. According to the current paradigm, a significant reduction in proton-pump activity at the plasma membrane would result either in cell death or a general down-regulation of all cellular functions. However, three lines of evidence indicate that these established functions were not signif-
significantly impacted by the loss of AHA10. First, the aha10 mutation was not lethal to seed coat endothelial cells, as evidenced by the production of brown-colored seeds. A lethal effect on this cell layer would be expected to produce a blond-colored seed (5). Second, mutant cells still produced normal or increased levels of non-PA pathway flavonoids such as anthocyanins and flavonols (Table 1). This observation is most easily explained as a specific block in the PA biosynthesis pathway, rather than a general starvation for initial precursors, which would be expected from a general reduction in nutrient and solute uptake across the plasma membrane. Third, the levels of two seed coat endothelium-specific mRNAs (BAN and TT12) are expressed at normal to increased levels in the mutant (Fig. 4). Thus, the seed coat endothelial cells in aha10 mutants appear to have normal plasma membrane functions that supply the cells with adequate nutrients and enable the signaling systems required for their differentiation as pigment-producing cells.

To explain the aha10 phenotype, we offer a model that posits a unique and direct effect of AHA10 on an endomembrane system. In this model, AHA10 still functions as a proton pump but is assigned to a known proton pump. The working model proposed above provides a logical explanation based on known activities of those pumps, P-type H^+-ATPases and P-type proton pumps, P-type H^+-ATPases and P-type proton pumps. However, regardless of the mechanism, the aha10/it phenotype is significant because it provides genetic evidence for an unexpected endomembrane function for a member of the plasma membrane proton-pump family. Although it is not clear whether AHA10 is the only member of this family to have an endomembrane function, our results support the hypothesis that some plant cells may use a P-type proton pump to help acidify endosomal or vacuolar compartments, in contrast to animal cells, which rely exclusively on V-type pumps.

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