Short title: 3D imaging P-type H\textsuperscript{+}-ATPase in Arabidopsis roots

Title: Environmental and Genetic Factors Regulating Localization of the Plant Plasma Membrane H\textsuperscript{+}-ATPase

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One Sentence Summary: Cellular dynamics and localization of plasma membrane H\textsuperscript{+}-ATPase are determined by cell type, light condition, and a receptor kinase, FERONIA that regulates cell elongation growth.

Author Contribution: M.H. and M.R.S. designed the experiments. M.H. performed all molecular cloning and plant work. M.H. and S.J.S. operated the microscope and captured images. L.X.T., D.B.B., and M.H. analyzed the image data. D.B.B. coded all of programs. M.H. interpreted biological results with discussion with L.X.T., D.B.B., and S.J.S. M.H. wrote the manuscript with M.R.S. L.X.T. and D.B.B. provided the text of the methods used for the data analyses. All authors participated in reviewing and editing the manuscript.

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ABSTRACT

A P-type H\(^+\)-ATPase is the primary transporter that converts ATP to electrochemical energy at the plasma membrane of higher plants. Its product, the protonmotive force (PMF), is composed of an electrical potential and a pH gradient. Many studies have demonstrated that this PMF not only drives the secondary transporters required for nutrient uptake, but also plays a direct role in regulating cell expansion. Here, we have generated a transgenic Arabidopsis plant expressing H\(^+\)-ATPase isoform 2 (AHA2) that is translationally fused with a fluorescent protein and examined its cellular localization by live-cell microscopy. Using a three-dimensional imaging approach with seedlings grown for various times under a variety of light intensities, we demonstrate that AHA2 localization at the plasma membrane of root cells requires light. In dim light conditions, AHA2 is found in intracellular compartments, in addition to the plasma membrane. This localization profile was age dependent and specific to cell types found in the transition zone located between the meristem and elongation zones. The accumulation of AHA2 in intracellular compartments is consistent with reduced H\(^+\) secretion near the transition zone and the suppression of root growth. By examining AHA2 localization in a knockout mutant of a receptor protein kinase, FERONIA (FER), we found that the intracellular accumulation of AHA2 in the transition zone is dependent on a functional FER-dependent inhibitory response in root elongation. Overall, this study provides a molecular underpinning for understanding the genetic, environmental, and developmental factors influencing root growth via localization of the plasma membrane H\(^+\)-ATPase.
INTRODUCTION

A unique feature of the plasma membrane in plants and fungi compared to animals is the proton electrochemical gradient produced by a P-type H\(^{+}\)-ATPase (proton pump), rather than by a sodium pump. Because the proton pump is moving only one proton per ATP molecule, the proton reversal potential of the enzyme is much larger than that of the sodium/potassium pump which moves three sodium ions out of a cell for every two potassium ions moving in. In concert with this intrinsic biochemical difference between the two enzymes, the membrane potential of plants is routinely found to be much more negative than in animals. It has allowed plants to evolve rapid hydraulic based methods for organ movement, such as is readily apparent in touch-sensitive plants, e.g. *Mimosa pudica* (sensitive plant) and *Dionaea muscipula* (venus flytrap). However all plants, even the slower growth movements that underlie plant specific phenonema such as photo and gravitropism, also rely on the deep membrane potential to ensure that the major osmolyte, the potassium ions (K\(^{+}\)), is concentrated in the cytoplasm and its movement across the membrane mediated by potassium channels ultimately induces the changes in cellular turgor pressure. The proton pump is also involved in tight regulation of the cell wall pH. This apoplastic pH in turn controls the extensibility of polymers in the wall, and is known to be altered rapidly and to different extents in different cell types within one organ, such as the root (Sussman and Harper, 1989; Michelet and Boutry, 1995; Palmgren, 2001).

The Arabidopsis genome contains a protein family of 100-kDa plasma membrane H\(^{+}\)-ATPases (AHAs) with 11 members, although AHA1 and AHA2 are the most predominantly expressed and play essential roles throughout the life cycle (Baxter et al., 2003; Haruta et al., 2010). During the seedling stage, AHA2 plays critical roles in establishing the proton gradient at the root surface as *aha2* insertion mutants show impaired proton secretion and root elongation at high apoplastic potassium concentrations (Fuglsang et al., 2007; Santi and Schmidt, 2009; Haruta and Sussman, 2012). A number of studies indicate that proton pump function is modulated at many levels, including transcriptional, translational, and posttranslational regulation (Duby and Boutry, 2009; Wang et al., 2014; Haruta et al., 2015; Falhof et al., 2016). In terms of catalytic activity, phosphorylation of regulatory serine and threonine residues modulate H\(^{+}\)-ATPase mediated proton pumping. Both genetic and biochemical analyses suggest that phosphorylation/dephosphorylation of the penultimate threonine residue (i.e., Thr947 of AHA2...
and Thr948 of AHA1 and 3) is key for regulating the ATPase by blue light and auxin (Fuglsang et al., 1999; Kinoshita and Shimazaki, 1999; Robertson et al., 2004; Takahashi et al., 2012; Spartz et al., 2014). Additional phosphorylation sites, such as Thr881 and Ser899 of AHA2, are also likely involved in the activity modulation and these may be independently regulated by various protein kinases and phosphatases that function in the signaling pathways initiated by receptor kinases such as FERONIA and PSY1R, in response to the cognate peptide ligands (Fuglsang et al., 2014; Haruta et al., 2014). The complexity of H⁺-ATPase regulation is further underscored by the recent observation that dephosphorylation at Thr947 of AHA2 by a protein phosphatase, PP2C plays an important and unique role in growth regulation by proteins encoded by auxin-induced SAURs (small auxin upregulated genes), suggesting that efforts in characterizing kinase-mediated phosphorylation may only be providing a partial understanding of the regulation of this enzyme (Spartz et al., 2014).

Localization of the H⁺-ATPase protein at the cell surface has been extensively characterized primarily using biochemical approaches. For example, plasma membrane fractions have been purified by enrichments using two-phase partitioning and/or by buoyant density gradient centrifugation (Scherer and Fischer, 1985; Sze, 1985; Larsson et al., 1987). Using these cellular fractionation methods combined with the enzyme activity assay, immuno-blots, or peptide sequencing via mass spectrometry, the H⁺-ATPase is often utilized as a common marker protein for the plasma membrane, although its presence only in the plasma membrane has never been critically tested under all growth conditions (Palmgren, 1990; DeWitt et al., 1996; Nelson et al., 2006). Tissue or cell-type specificity of H⁺-ATPase localization was shown via an immunocytochemical approach which allowed microscopic visualization of this protein at the surface of pollen, roots, and phloem companion cells (Parets-Soler et al., 1990; Obermeyer G, 1992; Samuels et al., 1992; Bouche-Pillon et al., 1994; DeWitt and Sussman, 1995). In a more recent study, GFP-tagged AHA1 overexpressed in the stomatal guard cells was visualized at the cell surface and its localization was partially dependent on a MUN domain protein, Munc13 (Hashimoto-Sugimoto et al., 2013). In another study, the level of AHA2-GFP protein expression in the primary root was shown to be altered when external nitrogen supplies were limiting (Mlodzinska et al., 2015).
Fluorescent microscope techniques combined with \textit{in vivo} expression of fluorescently labeled proteins or organelle staining dyes have been widely used to quantify protein localization and cellular dynamics (Lippincott-Schwartz et al., 2001; Hamilton, 2009). By capturing a series of confocal optical sections and rendering a projection, three dimensional views of live cells or tissues as well as localization patterns of a protein of interest can be obtained in a non-invasive manner (Masters et al., 1997; Truernit et al., 2008). Imaging developing Arabidopsis organs and quantifying fluorescent signals allow plant biologists to track cell lineage in addition to observing protein dynamics thus enabling computational modelling of biological processes (Roeder et al., 2011). In this study, we established 3D live-cell imaging tools and platform to examine the localization of AHA2 in an \textit{aha2-4} mutant line that stably expresses fluorescent protein (FP) tagged AHA2 under the native ATPase promoter. We examined how light affects FP-tagged AHA2 localization in the root and our results demonstrate that, under dim light conditions, FP-tagged AHA2 proteins accumulate in the cytoplasmic compartments in the cells residing in the transition zone. This localization change in AHA2 correlates with apoplastic pH alkalinization and root growth suppression. Furthermore, we show that this internal localization of AHA2 in the cytoplasm was dependent on the normal function of FERONIA receptor protein kinase. These results emphasize the importance of live-cell imaging with the plasma membrane proton pump, to understand membrane energetics and growth regulation \textit{in planta}.

\section*{RESULTS}

\subsection*{Expression of a mCitrine-AHA2 (mCitAHA2) Translational Fusion Protein}

To perform live-cell imaging of the AHA2 protein in Arabidopsis plants, we transformed \textit{aha2-4} insertional knockout mutant plants with an AHA2 genomic construct containing mCitrine fluorescent tag fused at the amino acid position, Leu4 of AHA2 (mCitAHA2, Fig. 1A). Two independently transformed mCitAHA2 lines, \textit{aha2-4;mCitAHA2a} and \textit{b} that are homozygous for a single insertion of the transgene, were used for further study. Our previous results have showed that the \textit{aha2-4} insertion mutants display impaired root elongation when grown on media containing high external potassium ion concentration, presumably due to the stress placed on energetics at the plasma membrane in the presence of high concentrations of this positively charged current-carrying abundant osmolyte (Haruta and Sussman, 2012). A comparison of \textit{aha2-4;AHA2} (complementation by wildtype AHA2 sequence) and \textit{aha2-4;mCitAHA2} lines in
the root growth assay indicates that mCitAHA2 can partially rescue the shorter root phenotype of 
aha2-4 (Fig. 1B). Together with the carboxyl terminus, the amino terminus of AHA2 is known to 
be a regulatory domain of its enzymatic activity (Ekberg et al., 2010), and thus the reduced 
ability of mCitAHA2 to rescue the impaired root growth of aha2 mutant is likely caused by 
misregulation due to compromising the intact amino terminus. To examine cellular localization 
of mCitAHA2 protein, we extracted and fractionated seedling tissues from aha2-4;mCitAHA2 
line to enrich for plasma membrane proteins (Fig. 1C). Immuno-blot detection of the soluble, 
endomembrane (lower phase from the two phase partitioning), and plasma membrane fractions 
(upper phase from the two phase partitioning) indicates that mCitAHA2 is enriched in the plasma 
membrane fraction of seedling extracts. The SDS-gel banding pattern suggests that mCitAHA2 
protein is present mainly in a monomeric form at ~125 kDa, with possible oligomers which were 
visible at higher molecular weight regions. The lower phase which contains primarily 
endomembranes such as the tonoplast, also contained mCitAHA2 protein. This may reflect two 
possibilities: (1) either the fractionation is not complete and thus PM-localized mCitAHA2 enter 
the lower phase in this protocol, or (2) mCitAHA2 protein is normally present in the 
endomembrane system, albeit to a smaller extent than found at the cell surface. Using live cell 
imaging we were able to use an independent orthogonal technology to examine this question.

**Correlation of Root Growth Suppression and Intracellular Accumulation of AHA2 under**

**Dim Light**

The H^+-ATPase plays a critical role in providing the energy for organ development and 
elongation processes during germination. For example, it is well known that seedling root and 
hypocotyl elongation is strongly influenced by the light intensity (Fankhauser and Chory, 1997). 
Light regulation of the H^+-ATPase activity is thought to contribute to optimizing growth rates of 
seedling organs (Spalding and Cosgrove, 1992; Takahashi et al., 2012) although a precise 
understanding of this process has not yet been achieved. To evaluate AHA2 localization at the 
cellular level, fluorescent signals of mCitAHA2 were visualized in seedlings grown for 10 days 
under low (2.2 μmol m^{-2} s^{-1}) or bright light (~10 μmol m^{-2} s^{-1}) intensities, using a Confocal Laser 
Scanning Microscopy (CLSM). Consistent with previously described typical Arabidopsis growth 
phenotypes at various light intensities (Hardtke and Deng, 2000; Chory, 2010), aha2-
4;mCitAHA2 roots grow longer in the bright light compared with dim light whereas hypocotyls
grew longer in the dim light condition compared with bright light (Fig. 2A). In order to visualize mCitAHA2 fluorescent signals in the tissues in which rapid cell growth is occurring, three-dimensional images were reconstructed from ~60 optical slices for the apex of hypocotyls and root tips (Fig. 2B-E). Under both the dim and bright light conditions, mCitAHA2 signals in hypocotyl cells were detected at the plasma membrane (Fig. 1B, C). In roots, mCitAHA2 was localized at the cell surface in the seedlings grown under the bright light condition (Fig. 2D). The seedling roots grown under the dim light showed the cell-surface localization of mCitAHA2 signal in the root tip as well as elongation zone but surprisingly, we observed accumulation of the signal in the cytoplasm of the cells in the transition zone (Fig. 2E). Thus, the shorter root length of the seedlings grown under the dim light correlates with the accumulation of mCitAHA2 in the cytoplasm in this region.

To further investigate the mCitAHA2 localization pattern with seedlings grown under the dim light condition, root growth was measured daily (Fig. 2F). A comparison of root growth curves under bright and dim conditions for a nine-day period shows that roots grown under the bright condition sustain growth whereas roots under the dim condition deviate from this and stop elongation around ~8 days old (Supplemental Fig. S1). Since the growth medium contains half-strength Murashige & Skoog nutrient salts and 1% sucrose, the root growth cessation under the dim condition is likely due to the endogenous developmental program instead of nutrient depletion. In rapidly growing roots at 3-5 days old under dim light, mCitAHA2 is localized at the plasma membrane (Fig. 2G), whereas the accumulation of mCitAHA2 in the cytoplasm was visible with 10-day-old seedlings (Fig. 2H). This cytoplasmic accumulation of mCitAHA2 was specific to cells residing in the transition zone of roots (Supplemental Movie 1).

The mCitAHA2 intensity levels were much greater at the plasma membrane compared to accumulation in cytoplasmic areas. This difference provided a means to quantify cytoplasmic and membrane accumulation (Fig. 2I, Supplemental Fig. S2). Voxels were divided by intensity into low and high intensity levels. The threshold for low intensity voxels was set above background but below the intensity levels occurring at the membrane. The threshold for high intensity voxels was set such that these voxels predominately occurred at the membrane. The total number of low (red) and high (yellow) intensity voxels were summed in windows 8 μm wide along the root length throughout the volume and divided by the total number of voxels above the background within the volume to give the ratio of voxels falling into either category.
In the roots grown under the dim light, at 80-140 μm from the tip, corresponding to the transition zone, a majority of voxels contained the low (red) intensity voxels indicating that mCitAHA2 was diffused in the cytoplasm. In contrast, in bright conditions, high intensity voxels occur much more frequently indicating dense mCitAHA2 accumulation at the cytoplasmic compartments. This effect is best summarized by subtracting the number of low intensity voxels from the high intensity voxels producing an accumulation index (blue). In the transition zone, in dim conditions, more than >80% of the voxels have low intensity but in comparison in bright conditions the accumulation index reaches almost 0 % indicating the number of high and low intensity voxels are equal. Additional experiments testing mCitAHA2 localization under various light intensity conditions ranging between 1.2 – 20 μmol m⁻² s⁻¹ found that the intensity at about 5 μmol m⁻² s⁻¹ is the critical point at which intracellular accumulation of mCitAHA1 occurs (Supplemental Fig. S3).

In separate experiments, we have also produced a different set of transgenic lines, aha2;Dendra2AHA2 plants that express Dendra2 fluorescent protein-tagged AHA2 in the aha2 mutant background (Supplemental Fig. S4A, B). The roots of aha2;Dendra2AHA2 seedlings grown under the bright or dim light conditions showed this FP-tagged AHA2 localization profile very similar to that seen with the aha2-4;mCitAHA2 plants, i.e., cell surface localization under the bright condition and intracellular accumulation in the transition zone of the roots under the dim condition. Those results support our observation that dim light caused the intracellular accumulation of AHA2 protein in the transition zone and that this was not due to a particular reporter protein used for the detection.

Characterization of Intracellular Accumulation of mCitAHA2 in the Transition Zone of Roots Grown under Dim Light

To quantitatively describe the intracellular localization of mCitAHA2 in the roots grown under dim light condition, we computationally segmented the 3D image of mCitAHA2 roots (Supplemental Fig. S5) into three parts: 1) meristem, 2) transition zone, and 3) elongation zone. The number of mCitAHA2-positive intracellular structures are quantified as detailed in Materials and Methods (Fig. 3A). At four days old, the number of intracellular structures is comparable among the three root zones. However, it was evident that at 11 days old, the mCitAHA2-labeled intracellular structures specifically accumulated in the transition zone, but not in the meristem or
elongation zone. Analyses for colocalization efficiencies of mCitAHA2 signals and the surface of live cells stained with impermeant propidium iodide (PI) revealed that the group of cells residing in the transition zone had significantly lower $R^2$ regression values than those in the elongation and meristem regions (Fig. 3D). The relative lower $R^2$ value for the transition zone agreed with our visual inspection of the mCitAHA2 accumulation in the cytoplasm of the cells in the transition zone since the cell surface was clearly visible in the PI channel (Fig. 3E, F).

Examination of the XY section of the 3D image suggested that mCitAHA2 signal accumulated in intracellular components as large vesicles (Fig. 3G, H). In the cells that are experiencing elongation, mCitAHA2 signals were diffused across the cells. At the maturation zone, a majority of mCitAHA2 signals were localized to the plasma membrane and no detectable signals in the intracellular space (Fig. 3H-J). Those images may be reflecting the localization of AHA2 protein molecules that were in the process of trafficking to or from the PM and were paused in their locations when the cell elongation process was inhibited due to the absence of sufficient light intensity.

Intracellular Accumulation of mCitAHA2 Correlates with pH Alkalinization at the Root Surface of the Transition Zone

Both our previous experiments and other studies have shown that epidermal cells of the primary root contain a high expression level of the AHA2 transcript and protein, and this expression pattern was supported by impaired root growth phenotypes of $aha2$ mutants on appropriate growth conditions (Haruta and Sussman, 2012; Mlodzinska et al., 2015). Thus proton secretion by AHA2 activity is expected to play the major role in creating proton gradient at the root surface. To examine proton concentrations at the surface of roots grown under dim light, we surrounded mCitAHA2 seedling roots in a solution containing a pH probe, fluorescein-dextran (FITC-dextran), and captured 2D images (Fig. 4A). FITC dextran has been used to visualize pH changes at the root surface in Arabidopsis (Ohkuma and Poole, 1978; Monshausen et al., 2007). Surface pH was calibrated with standard solutions buffered to pH 5.5, 6.0, or 6.5 (Supplemental Fig. S6). Consistent with the above observation, mCitAHA2 protein clearly localized at the PM in the maturation and meristematic zones but accumulated in the cytoplasmic compartments in the transition zone. Signals from the FITC pH reporter at the root surface and its profiling reveal higher pH values in the apoplast of the transition zone compared with that in the maturation zone.
(Fig. 4B-D), correlating with reduced abundance of mCitAHA2 protein in the plasma membrane in this region. Unexpectedly, we also noticed that this higher pH band spans above the transition zone to the elongation zone, suggesting that the H$^+$-ATPase in this region is less active possibly caused by less 14-3-3 binding, altered phosphorylation, or low cytosolic pH. Alternatively, it may be caused by changes in other proton transporters (e.g., proton coupled carriers) or other pH regulating mechanisms in the elongation zone (see discussion).

As a control, we tested the effect of buffered solutions (pH 5.5) containing the pH probe to ensure that changes in the FITC fluorescent intensity are responding to pH values and not to changes in other ion concentrations (Fig. 4E-H). In these experiments, the bright green signals seen at the transition zone of the root submerged in the unbuffered solution disappeared in the buffered medium, supporting the idea that our observations using unbuffered media indeed reports changes in pH values.

FERONIA Receptor Kinase is Required for Root Growth Suppression and mCitAHA2 Intracellular Accumulation in Seedling Roots Grown under Dim Light

During seedling development, root growth is presumably optimized by the fine adjustment of positive and negative regulatory mechanisms. Our model predicts that insufficient light intensities will suppress root elongation and this is in part mediated by an intracellular signaling cascade initiated by the FERONIA receptor kinase (Haruta et al., 2014). This idea is supported by loss-of-function mutants of FERONIA that show faster root elongation under dim light condition whereas wildtype root growth is suppressed in the same dim light condition (Haruta et al., 2014). To further compare spatial regulation of mCitAHA2 protein between wildtype and fer mutant plants, we produced fer-4;aha2-4 double mutants carrying the mCitAHA2 fluorescent reporter by genetic crossing of fer-4 and aha2-4;mCitAHA2a or b. Under the bright light condition (10 μmol/m$^2$/sec), root length measurements of aha2-4;mCitAHA2 and fer-4;aha2-4;mCitAHA2 are comparable (Fig. 5A, C). As the light intensity decreases, aha2-4;mCitAHA2 root length was dramatically decreased when measured at 10 days old (Fig. 5A, B). Root growth measurements of fer-4;aha2-4;mCitAHA2 seedlings were also reduced as the light intensity decreases and the effect of fer-4 knockout mutation was clearly visible since fer-4;aha2-4;mCitAHA2 roots grow faster than aha2-4;mCitAHA2 roots.

The roots of seedlings carrying a wildtype FER allele show the accumulation of
mCitAHA2 fluorescent signals in cytoplasmic compartments of specific cells under the dim light conditions (2.2 μmol/m²/sec). Fluorescent intensity profiling of mCitAHA2 in the aha2-4;mCitAHA2 seedling grown under the dim light was performed with a method similar to that shown in Fig. 2I, and these displayed a distinct profile from that in the same genotype grown under bright light (Fig. 5D, E). In contrast, under dim light conditions mCitAHA2 localization profiles in the fer-4;aha2-4;mCitAHA2 roots appeared to resemble those observed with the same genotype grown under the bright light (Fig. 5F, G). Because mCitAHA2 signals in the plants with fer-4 genetic background appear to be lower than those with FER4 wildtype plants (Fig. 5F,G), we extracted total protein from seedlings grown under bright or dim conditions and examined mCitAHA2 protein abundance with GFP immuno blot (Fig. 5H, I). Whereas mCitAHA2 level is comparable between the two genotypes when plants were grown under bright light, mCitAHA2 protein level in plants with fer-4 allele is somehow higher than in those with FER wildtype allele when plants were grown under dim light (Fig. 5I). This observation seems to be contradictory with the results seen with mCitAHA2 fluorescent imaging (Fig. 5F,G); however, it is likely that the results from immuno blot assay reflects true mCitAHA2 protein abundance as fer-4 roots secrets more protons to the apoplast and elongates faster than wildtype (Haruta et al., 2014; Du et al., 2016; Yeats et al., 2016)(see Discussion for further description). Regardless weak mCitrine fluorescent signals in fer-4 genetic background, overall these results are consistent with the faster growth of fer-4 mutant roots being caused by the absence of FERONIA kinase suppressing the intracellular accumulation of mCitAHA2 protein under the dim light condition, leading to increased proton pump function and an increased rate of cell expansion.

Discussion

In this study, we investigated when and where plasma membrane H⁺-ATPase function is regulated at cellular level in Arabidopsis roots. The H⁺-ATPase is long known to be localized at the plasma membrane as this enzyme fulfills the essential role in secreting protons into the apoplast to generate both charge and pH gradients necessary to energize nutrient uptake. However, the dynamic regulation of pump localization at the cellular and subcellular levels is poorly understood. By visualizing fluorescently labeled AHA2 protein with a 3D live-cell imaging approach, we have found that AHA2 localization in roots is influenced by light during
elongating growth, which is specifically observed in a particular region of the root, the transition zone. We also demonstrate that this light- and cell-type dependent localization of AHA2 requires the function of the FERONIA receptor kinase localized at the plasma membrane.

**Fluorescent Fusion Protein of AHA2 Reports Its Cellular Localization in Live Cells**

Our challenge in the understanding H⁺-ATPase regulation at cellular localization level has been, in part, due to the absence of an appropriate genetic resource that can report on membrane trafficking processes of the H⁺-ATPase *in vivo*. We have attempted to produce Arabidopsis lines expressing a genetically encoded fusion protein of AHA2 with fluorescent proteins, by complementing *aha2* insertion mutant phenotype using the native promotor. Whereas not all the fluorophores tested here provided robust and reliable signals, we confirmed that mCitrine, which is a yellow derivative of a jelly fish green fluorescent protein (GFP), and Dendra2, which is a derivative of a coral fluorescent protein (FP), reported similar AHA2 localization patterns in the roots of developing seedlings. In our root growth phenotyping assay these fluorescently tagged AHA2 constructs can rescue the *aha2-4* mutant phenotype only partially; however, FP-tagged AHA2 localization changes in response to the growth environment and genetic ablation indicate that the signals from the FP-tagged AHA2 proteins likely reflect *in vivo* behavior of the H⁺-ATPase molecule. The importance of choosing appropriate fluorophores and protein tagging sites within protein sequence was also suggested from an FP-tagging study of vacuolar H⁺-pyrophosphatase, which is also a polytopic transmembrane protein (Segami et al., 2014).

In our control experiment, we grew an Arabidopsis line expressing GFP-tagged ATP-binding cassette transporter 36, ABCG36 (PEN3), known to be localized at the plasma membrane and proposed to transport a wide range of solutes to the apoplast, under identical conditions as the FP-tagged AHA2 (Supplemental Fig. S6; (Stein et al., 2006; Kim et al., 2007). This protein, however, was not accumulated in intracellular compartments, unlike the mCitAHA2 and Dendra2AHA2, thus indicating that not all of the plasma membrane proteins undergo intracellular accumulation under the dim light growth condition. AHA2 is preferentially localized at the top and bottom of epidermal cells in the transition to elongation zones, whereas consistent with the previous study, ABCG36/PEN3 is localized at the outer edge of the epidermal cells in those regions (Supplemental Fig. 7A, B; (Strader and Bartel, 2009). Thus it is possible that the mCitAHA2 molecules that were normally targeted to the PM at the top or
bottom of cells under the bright light conditions were internalized to down regulate AHA2
function in the absence of sufficient light intensities. This effect of light could be to either
increase the movement of vesicles to, or decrease the movement from, the plasma membrane
since our experiments cannot distinguish between these two processes.

A previous study of the plasma membrane H⁺-ATPase movement to and from the plasma
membrane has reported that indole acetic acid (IAA) treatment enhanced membrane flow from
the endoplasmic reticulum to the plasma membrane (Hager et al., 1991). The IAA-induced
increase in the H⁺-ATPase protein level at the plasma membrane was observed within 10 min,
which correlated with a decrease in the pH value of bathing medium and the initiation of the
elongation growth of maize coleoptiles. This study utilized coleoptiles of maize and thus together
with the present study using Arabidopsis, our collective results support a critical role of the H⁺-
ATPase trafficking dynamics between the endomembrane and plasma membrane in the
regulation of elongation growth of plant organs.

Seedling Roots Adapt to Dim Light Conditions by Intracellular Accumulation of AHA2,
Cell Surface pH Alkalinization in the Transition Zone, and Growth Suppression
The transition zone has also been referred to previously as the distal elongation zone (DEZ), in
which roots initiate responses to various stimuli such as the gravity and mechanical force
(Ishikawa and Evans, 1995). More recent studies suggest that the cells in this region are more
active in ion transport processes compared with the cells in regions above and below (Masi et al.,
2009). Our observations of the transition zone acting as the important growth regulatory region
for localizing the AHA2 protein at the PM coincide with the previously noted distinctive roles of
the cells in this region in determining root physiology; however, there are no causal links to
specifically relate the down regulation of AHA2 function in this region and the previous
observations reporting on the role of the transition zone in environmental sensing. Interestingly,
Wan et al. showed that another plasma membrane transporter, PIN2, known to function in auxin
transport processes, was accumulated in intracellular compartments in the area covering the
transition zone in dark-grown roots (Wan et al., 2012). The authors of this study and another
earlier study proposed vacuole-like compartments (VLCs) or prevacuolar compartment (PVC) as
possible organelles in which PIN2 accumulates when seedlings were grown under the dark
condition (Kleine-Vehn et al., 2008). Since AHA2 is the major cation transporting protein at the
plasma membrane during longitudinal growth of roots, it would not be surprising if other anionic
solute transporters such as PIN2 are also subject to the reduced accumulation within the same
PM compartment. Although it remains to be determined whether AHA2 and PIN2 are trafficked
in the same or different types of vesicles/organelles, one may speculate that AHA2 also
accumulates or is subject to proteolysis in the VLCs or PVC in the transition zone of roots grown
under dim light. This notion is also supported by a potential truncation of mCitrine tag in the
seedling grown under dim light (Fig. 5H).

Our pH measurement assay shows an overall correlative trend between higher surface pH
values and intracellular accumulation of mCitAHA2 protein in the transition zone of the roots
grown under dim light. This supports our model that AHA2 proton secretion activity in this
region is a major contributor to the regulation of the proton motive force and root growth. This
idea is also supported by a recent observation that acidic apoplastic pH contributes to cell
expansion in roots (Barbez et al., 2017). We did not identify possible causes of a stretch of
higher pH region also observed above the transition zone. One possibility is that the alkaline pH
observed here was caused by a diffusion of the anionic form of the pH reporter, FITC, from the
adjacent transition zone during the experiment, thus resulting in bright fluorescent signals near
the elongation zone. The use of an apoplastic pH reporter system that is based on a genetically
encoded pH sensitive fluorophore may also help clarify our observation of the alkaline pH above
the transition zone (Gjetting et al., 2012). Alternatively, the use of another pH reporter dye for
ratio measurements helps correct a potential inhomogeneous concentration errors observed with
the FITC reporter. Other plausible explanations are that the transport of ions other than protons
may also contribute to the alkalization seen at the cell surface. Alternatively, proton-coupled
solute transporters could be more active at the region and cause a higher rate of proton import
into these cells. If the latter is the case, in the rapidly expanding cells nutrients are likely actively
taken up into the cells together with protons (Yeats et al., 2016), thus depleting apoplastic proton
concentration and inducing the external pH alkalization in the absence of AHA2 function or in
conjunction with the reduction of proton extrusion by AHA2.

FERONIA Receptor Kinase Acts Upstream AHA2 in a Signal Pathway That Restricts
Seedling Root Elongation Under Dim Light Condition

During germination and seedling stages, the preformed cotyledon and root stored in seeds
contributes to provide nutrient and energy to drive expansion growth. The reserved nutrients would last until true leaves emerge during postembryonic development, which occurs at approximately 10 days after germination (Boyes et al., 2001). During this period, the rates of seed germination and seedling growth are highly influenced by light intensity. Our observation that the intracellular accumulation of mCitAHA2 became visible at approximately 10 days old when grown under dim light, correlates with the timing of the onset of this postembryonic development. We predict that dim-light grown seedlings which did not establish photosynthetic capability cease root growth at this age, which coincides with the removal of the H\textsuperscript{+}-ATPase from the plasma membrane in the cells of the transition zone. Although our results do not provide any experimental evidence as to how seedling roots sense light intensity or transmit light signaling, previous studies showed that blue light receptors, cryptochrome and phototropin are both expressed in seedling roots (Sakamoto and Briggs, 2002; Endo et al., 2007). Thus it is possible that seedling roots can directly sense light intensity and quality. We did not test whether the intracellular accumulation of mCitAHA2 occurred in the seeds germinating on the soil surface; however, one could speculate a similar situation would occur when roots are crawling just near on the soil surface for the first several days after the germination and then seedling roots thus can directly sense light intensity to adapt to the light environment. Despite this speculation, it remains to be studied whether soil-grown adult plants respond in the similar way as seedlings did with the intracellular accumulation of mCitAHA2. In the adult plants grown in soil, there are at least several different light sensing mechanisms by which leaves and stems sense light and transmit signals to roots (Lee et al., 2017; van Gelderen et al., 2017).

In Arabidopsis roots, AHA2 activity appears to be regulated by protein phosphorylation pathways involving multiple protein kinases and phosphatases (Fuglsang et al., 2007; Nuhse et al., 2007; Spartz et al., 2014). FERONIA receptor kinase is one of the proteins regulating AHA2 through a transient phosphorylation at Ser899 site (Haruta et al., 2014). To support a genetic basis for FERONIA kinase as a regulator of AHA2, we examined mCitAHA2 fluorescent signals in fer-4;aha2-4 double mutant background and compared that in an aha2-4 single mutant. In agreement with the fer-4 root elongation phenotype, mCitAHA2 in seedling roots carrying fer-4 allele remained localized at the PM in the seedling roots grown under the dim-light growth condition whereas in roots carrying the FER wildtype allele, the pump is internalized (Fig. 5). This supports our model that FERONIA is acting upstream of AHA2 in the signaling pathway of
RALF and FERONIA-regulated root cell growth expansion. We have also noticed that mCitAHA2 fluorescent signals from fer-4;aha2-4 seedling roots were weaker than those from aha2-4 seedling roots. This was observed in the two independently transformed aha2-4;mCitAHA2 lines that are independently crossed to fer-4 mutant. Since fer-4 roots secrete more protons and elongate faster than wildtype (Haruta et al., 2014; Du et al., 2016), it is possible that fer-4 root cells accumulate more or less of other solutes, such as chloride anions, in the cytoplasm. Indirect measurements of the plasma membrane electric potential suggested that fer-4 root cells display a deeper electric potential (Yeats et al., 2016). Fluorescent intensity emitted by many fluorophores including mCitrine, is influenced by microenvironments including pH and ion concentrations surrounding the FP molecules (Griesbeck et al., 2001). Thus, if cytoplasmic ion(s) concentrations are altered in fer-4 mutant, we may observe increases or reduction of mCitAHA2 signals caused by this change. Regardless of the cause, mCitAHA2 localization profiles in fer-4 roots grown under the two light conditions are very distinct from those in FER wildtype genetic backgrounds, supporting our model that FERONIA action is located at the upstream of AHA2 function and influence its localization at the PM.

Conclusion

Altogether, we have produced imaging tools and methods to analyze spatial regulation of the plasma membrane H^+-ATPase molecule localization in vivo. The use of 3D imaging and intensity profiling approaches provide an unbiased view of AHA2 localization. Many previous studies have provided observations that support an acid growth model in which there is a positive correlation between H^+-ATPase activity and cell expansion (Rayle and Cleland, 1992; Hager, 2003). Although the model has been extensively examined and discussed, no single experimental system has shown live-cell measurement of apoplastic pH and H^+-ATPase localization in vivo. Our experiment uncovered differential localization of the H^+-ATPase protein within root regions, in which this protein is subject to accumulation in intracellular vesicles that cannot contribute to events at the plasma membrane surface. Together with the previous discussion from a study of high-resolution analysis of cell elongation control by auxin and the H^+-ATPase in hypocotyls (Fendrych et al., 2016), our result highlights the importance of the experimental approach for testing a causal relationship between H^+-ATPase activation/inactivation and cell growth in cell-type specific or single cell levels. Future development of a higher resolution imaging assay will
allow us to directly examine how individual H\(^+\)-ATPase proteins move within the plane of the membrane and overall, how their function influences cell elongation in planta.

MATERIALS AND METHODS

Plant Materials

*Arabidopsis thaliana* mutant plants, *aha2-4* and *feronia-4* (ecotype Columbia) were previously isolated and published (Haruta and Sussman, 2012; Haruta et al., 2014). PEN3-GFP seeds (CS67802) were obtained from Arabidopsis Biological Resource Center (ABRC; (Stein et al., 2006). Arabidopsis plants were grown at 22\(^\circ\)C with constant light and transformed by the floral dip method using the Agrobacterium strain, AGL1 (Clough and Bent, 1998).

Root Growth Assay

Arabidopsis seeds were plated with approximately 3 mm apart onto a half-strength MS medium (pH5.7) containing 1% sucrose and 0.8% agar. Seeds were stratified at 4\(^\circ\)C for 48 hr. Plates containing seeds were placed at the vertical orientation in a plastic box with blue translucent color. The entire box containing plates and seeds were placed onto the shelves in a growth incubator (Percival, Model I36LLVL) at 22\(^\circ\)C and illuminated with cool white light (GE F20T12 CW, Cool White, 20W). Detailed description of the experimental setting for bright (10 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) or dim (2.2 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) light conditions were provided in Supplemental Figure S8A. Light wavelengths transmitted through the blue colored boxes were measured with a spectrophotometer (SpectroSuite, Ocean Optics), resulting in a spectrum that was enriched in blue light ranges over red lights compared with the white light (Supplemental Figure S8B). The use of the light condition that was enriched with blue light ranges was justified as seedlings grown under this dim blue-enriched light condition grew more uniformly compared with those under dim white light, which was critical to minimize the variability in root length measurements and observe the consistent mCItAHA2 localization patterns.

For root growth assay with high potassium concentration, seeds were grown for three days at the vertical orientation under the constant white light. Seedlings were aseptically transferred to the control media or the media supplemented with 100 mM KCl. Plants were grown for additional 10 days. To quantify root length, plates containing plants were scanned with an office scanner and the measurements were carried out with ImageJ (Schneider et al., 2012).
Expression Plasmid DNA Construct

For expression of mCitrine-AHA2 translational fusion protein in planta, AHA2 genomic DNA fragment containing the native promoter, the entire gene, and 3’ regulatory sequences was used (Haruta et al., 2010). To produce mCitrine tag insertion site, a restriction site for Xba I was introduced at the amino acid position, Leu4, using the site-directed mutagenesis technique. Due to this large size of the plasmid containing genomic sequence of AHA2 (a total of ~20 kb), to facilitate the molecular cloning procedure, a small portion of AHA2 genomic sequence (2,602 kb) flanked by two restriction enzyme sites, Stu I and Pac I was subcloned in pCR2.1 (Invitrogen). This pCR-AHA2sp plasmid containing the Leu4 region was used for introducing a restriction site Xba I via site-directed mutagenesis using Change-IT mutagenesis kit (Affymetrix) and a primer, 5’-AGTGGTGAGAGATGTCGAGTCTAGAAGATATCAAGAACGA-3’.

Resulting AHA2 sequence containing the restriction site Xba I was excised from pCR2.1-AHA2sp plasmid by digesting with Stu I and Pac I and used to ligate into the plant expression vector carrying the genomic AHA2 sequence, pCAMBIA1200-gAHA2 (Haruta et al., 2010). Thus, wildtype DNA fragment containing the Leu4 position was replaced with the DNA fragment containing the Xba I restriction site. DNA sequence encoding mCitrine (a variant of yellow fluorescent protein, YFP) was produced by mutagenizing YFP sequence of pEarleyGate104 (CD3-686, ABRC; (Earley et al., 2006). To improve the function of YFP, two mutations, A206K (monomeric fluorescent protein)(von Stetten et al., 2012) and Q69M (photostability)(Griesbeck et al., 2001) were introduced by site-directed mutagenesis using two primers, 5’-/5Phos/CTGAGCTACCAGTCCAAACTGAGCAAAGACCCC-3’ and 5’-/5Phos/TTCGGCTACGCGCTGATGCTTCGCTGCCGCTA-3’. The mCitrine sequence flanked by Xba I was PCR-amplified using a pair of primers, 5’-
TCTAGAAAATGGTGAGCAAGGCGA GGAG-3’ and 5’-
TCTAGACTCTTTGTACAGCTCGTCCATGCC-3’ and used to ligate into the pCAMBIA1200-gAHA2XbaI to produce a construct for expressing mCitrine-AHA2 translational fusion under the native promoter in planta. Transgenic lines were selected on the media containing 25 μg/mL hygromycin and the transformation was validated by PCR-based genotyping with set of primers amplifying a region covering a junction of AHA2 and mCitrine sequences, 5’-
GAACCTAAAGAGCCAGCAA-3’ and 5’-TCTAGACTCTTTGTACAGCCTCGTCCATGCC-3’.
A plasmid carrying Dendra2 coding DNA was obtained from Addgene (#57701(McKinney et al., 2009). Dendra2 sequence was amplified using a pair of primers: 5’-
TCTAGAAACACCCGGGAATTAACCTG ATCAA-3’ and 5’-
TTCTAGACTCCACACCTGGCTGGCAGGAGGCTGT-3’. The amplified PCR fragment was
cloned into pCAMBIA1200-gAHA2XbaI. Transgenic lines were genotyped with a pair of
primers, 5’-GAACCTAAAGAGGCAGCAA-3’ and 5’-
TTCTAGACTCCACACCTGGCTGGCAGGAGGCTGT-3’.

Plasma Membrane Protein Extraction and Immuno Blotting

Arabidopsis seeds (10 mg) were grown in a magenta box containing 75 mL MS liquid media for
14 days under constant light. Tissues (~ 45g) were ground in a homogenizer with 150 mL
extraction buffer (100 mM Tris-HCl pH7.5, 300 mM sucrose, 25 mM EDTA, 25 mM NaF, 1
mM Na₂MoO₄, 1 mM PMSF, 1 mM DTT, 100 μM 1,10-phenanthroline) for 90 seconds at 4°C.
The homogenate was filtered through two layers of miracloth and spun at 1,000 x g for 10 min at
4°C. Supernatant was centrifuged at 100,000 x g for 35 min at 4°C to obtain the microsomal
pellet. The pellets were resuspended in suspension buffer (10 mM Tris-HCl, pH7.5, 300 mM
sucrose, 1 mM EDTA) and subjected to the two phase partition protocol to obtain the plasma
membrane enriched fraction (Larsson et al., 1987). The upper (PM) and lower (endomembrane)
phases were diluted two fold with the resuspension buffer and spun at 100,000 x g for 50 min for
4°C. The pellet was resuspended in the suspension buffer and stored at -80°C. Protein
concentration was determined by BCA assay (Pierce).

Soluble or membrane protein samples were adjusted to 15 ug in 30 uL and mixed with
0.1% Triton X-100. Protein samples were mixed with LDS sample buffer (novex, Life
Technologies) and Sample Reducing Agent (novex, Life Technologies). Protein solution was
heated at 70°C for 10 min. Protein sample was loaded onto a protein gel and resolved by running
at ~125 V for 45 min. Protein was transferred to a membrane filter (Immobilon FL, Merck
Millipore) using XCell II Blot module (Invitrogen). Membrane was blocked with Odyssey
blocking buffer (LI-COR) and probed with primary antibodies. GFP antibody (Genscript) was
diluted to 1:1000 with the blocking buffer and used to react with the epitopes. A secondary
antibody, goat anti-mouse IgG labeled with IRDye 680RD (LI-COR) was reacted with the blot.
Signal was visualized and recorded using Odyssey imaging system (LI-COR).
The identical blot membranes were stained with Ponceau S to visualize the proteins loaded after the detection of signals from the secondary antibody.

**Analysis of AHA2 Protein Abundance in Total Protein Extract from Seedlings**

Protein was extracted from seven 10-day-old seedlings grown under bright or dim light conditions. Seedlings were ground in 140 μL 50 mM HEPES buffer (pH7.5) containing 1X cOmplete Protease Inhibitor Cocktail (Roche) and 0.5% Triton X-100. The homogenate was spun at 12,000 rpm for 10 min at 4°C and the supernatant was recovered. Protein concentration was determined by BCA assay. Protein samples (7.5 μg/30 μL) were mixed with the LDS buffer and the reducing agent prior to heating at 70°C. Proteins were resolved by protein gel and mCitAHA2 was detected with GFP immuno blot as described above.

**Confocal Laser Scanning Microscopy for 3D Imaging of Roots**

Confocal microscope imaging was performed with Zeiss LSM780 ELYRA PS.1. Seedlings were submerged in water at the surface of a coverslip (48 X 60 mm, No 1, Gold Seal COVER GLASS). To visualize mCitAHA2 protein, the fluorophore was excited at 514 nm with 2.5-10% laser power and emission signal was detected at 520-620 nm. Image was viewed and captured, using an objective lens, LD C-Apochromat 40x/1.1 W Korr M27, unless otherwise noted. Three-dimensional imaging was performed by scanning ~60 optical slices of an Arabidopsis root, with an interval of ~1.06 micrometers for z-depth range of ~68 micrometers. Detector gain was typically set to 750-800. Before recording images, signal intensity across the entire view was manually inspected in order to prevent signal saturation. Images were acquired with line sequential mode and averaging of 4. A stack of optically sliced images were compiled to single 3D images using ZEN or ZEN lite 2012 software and presented with the maximum intensity projection protocol. Image was recorded with pinhole size 1.01 Airy Units (1.2 μm section), 512 X 512 pixel sizes, 8 bit depth, 3.15 us pixel time, 0.03 ms line time, and 7.75 s frame time. For the movie, 3D images (.czi file) of Arabidopsis roots obtained with the confocal microscope were animated with the ZEN software, exported to Quicktime movie format (.mov), and edited with Adobe Premiere Pro. For GFP or Dendra2 detection, the fluorophore was excited at 488 nm with 2.0% laser power percent and detected at 493-550 nm. For both mCitrine and GFP detection, wildtype non-transgenic seedlings were also viewed with the identical conditions to ensure that
any fluorescent signals detected with the mCitrine- or GFP-plants are not due to endogenous autofluorescence.

Quantitation of Intracellular Components and Colocalization Analysis

Images were analyzed with Imaris 7.6.1 (Bitplane). All images were subjected to background subtraction and Gaussian filtering prior to surface rendering with the ‘Surfaces’ tool. Each image was divided into 3 regions of interest: meristem (61.28 μm from tip), transition zone (63.77 μm above meristem), and elongation zone (86.95 μm above transition zone). Each of these regions was then subjected to surface rendering using histogram-based automatic thresholding. Because intracellular components appear vesicular, the ‘sphericity’ filter was applied to the rendered surfaces to select and quantify these structures. A manually selected ‘sphericity’ threshold of 0.67 (where a value of 1 is a perfect sphere) was applied and kept constant between images. The following data were extracted from the statistics tab of Imaris: number of spherical structures, fluorescence intensity, and number of disconnected components.

For co-localization analyses, images were similarly subjected to background subtraction and Gaussian filtering. The image was then segmented into 4 regions of interest. “Pearson’s coefficient in dataset volume” for each region was obtained from the “Coloc” tab of Imaris. R² values were calculated by obtaining the square of Pearson’s coefficient values (Dunn et al., 2011).

Fluorescent Intensity Profiling of mCitAHA2

Image files (.czi) were processed using Fiji software (Schindelin et al., 2012). The image files were converted to the .tif format and used for intensity profiling using custom scripts written in Python. When comparing mCitAHA2 levels the images were normalized by subtracting the minimum intensity from all pixels and then divided by the maximum pixel intensity. Intensity values range from 0 to 1, with 1 being the maximum intensity corresponding to maximum mCitAHA2 concentration. The voxels were divided into two distinct groups by intensity. The root regions that had intracellular accumulation of mCitAHA2 signals had reduced intensity since the H⁺-ATPase was spread over a larger volume. In contrast, mCitAHA2 localization to the membrane concentrated the protein into smaller volume producing high intensity voxels. Within the images an intensity ranging from 0.1 -0.35 occurred predominately within the intracellular
space whereas intensities > 0.35 occurred predominately at the membrane. When summing up
the number of voxels along the length of the root, the volume includes 8 μm below and above the
given location. The number of voxels at a given intensity level were divided by the total number
of voxels above background to control for differences in root size. For the data shown in Fig. 2I,
quantitation was performed using three images per condition and presented with the mean with
the standard error. Since the triplicated image analysis produced an intensity profile that is very
similar to the individual images, for Fig. 5D-G, Supplemental Fig. S1., S3, intensity profiling
was performed from one imaging data per condition as a representation of multiple images (n>3
per condition) which showed similar localization patterns. All the programs developed in this
study is available at the GitHub code repository site (https://github.com/DanBushey/Image-

Root Surface pH Measurement

A ten-day-old seedling grown on a half-strength MS media at vertical position was submerged in
20 μL solution containing 1 mM CaCl₂, 1 mM KCl and 3 mg/mL fluorescein isothiocyanate
dextran (FITC-dextran, average molecular weight 10,000, Sigma FD10s) on the surface of a
cover slip. Image was acquired with a 20X objective (Plan-Apochromat 20x/0.8 M27), image
size 1024 x 1024 pixels, 16 bit depth, averaging 16. FITC-dextran was excited at 488 nm with
laser power 1.8% and detected at emission wavelengths, 493-597 nm. Detector gain was set to
400. Images were recorded with 0.64 us pixel time, 0.35 us, line time, and 25.02 s frame time.
The pH standard solutions were composed of 10 mM MES, 1 mM CaCl₂, and 1 mM KCl, and
adjusted pH values to 5.0, 5.5, 6.0 or 6.5. FITC-dextran were added to the pH standard solutions
at 3 mg/mL immediately before imaging. Images for the pH standard solutions were acquired
using the identical method with the apoplastic pH measurement described above.

The regression analysis was performed to examine and generate a pH standard curve. A
liner fit was obtained with the data points of pH5.5, 6.0, and 6.5. The slope was used to
determine pH of the root surface. A region of interest (ROI) including the periphery of the root
was generated through an automated script. The dye did not penetrate the root which allowed a
maximum threshold to be set that separated the root from the surrounding fluorescent medium.
The mask including the root was dilated by an area of 50 pixels. An ROI including only the
periphery was generated by subtracting the dilated mask from the original root mask. Mean
intensity was calculated along the root length in an area 8 μm wide. ROI generation and
calculations were performed using scripts written in Python.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Root growth curve of aha2-4;mCitAHA2 roots grown under bright or
dim light conditions.

Supplemental Figure S2. Raw root images that are used for mCitAHA2 fluorescent intensity
profiling.

Supplemental Figure S3. Effect of a series of light intensities on mCitAHA2 localization.

Supplemental Figure S4. Localization and intensity profiling of Dendra2AHA2 protein.

Supplemental Figure S5. Segmentation of 3D image obtained with aha2-4;mCitAHA2 root.

Supplemental Figure S6. Calibration of a pH probe, FITC-dextran.

Supplemental Figure S7. Localization of mCitAHA2 and PEN3-GFP in the seedlings grown
under dim light.

Supplemental Figure S8. Description of light conditions for seedling growth assay.

Supplemental Movie 1. 3D image of the transition zone of aha2-4;mCitAHA2 root grown under
dim light.

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FIGURE LEGENDS

Figure 1. Expression of fluorescently labeled AHA2 in plants. A, Membrane topology of
mCitrineAHA2 (mCitAHA2) fusion protein. The mCitrine fluorescent protein sequence was
inserted at the amino acid position Leu4 located in the cytoplasmic face of AHA2 sequence. B,
Root growth measurements of mCitAHA2 expressing plants. Arabidopsis seedlings of wildtype, aha2-4 mutant, aha2-4;AHA2 (wildtype complementation) or aha2-4;mCitAHA2 were grown for three days onto 1/2MS media, then transferred to the media supplemented with 100 mM KCl, and grown for additional 10 days. C, Immunological detection of mCitAHA2 protein from aha2-4;mCitAHA2 plants. Ten-day-old seedlings were extracted to enrich the plasma membrane fraction using the two-phase partition method. Sol., fraction containing soluble proteins. LP, lower phase fraction containing endomembrane proteins. UP, upper phase fraction containing plasma membrane proteins. Fifteen microgram of proteins were loaded onto protein gel. Blot was probed with anti-GFP (Right). Once the blot membranes were scanned for the immuno detection, they were stained with Ponceau S to visualize proteins (Left).

Figure 2. Organ length and mCitAHA2 localization in aha2-4;mCitAHA2 plants grown under bright or dim light conditions. A, Hypocotyl and root length of aha2-4;mCitAHA2 seedlings grown for 10 days under dim (2.2 μmol m⁻² s⁻¹) or bright (10 μmol m⁻² s⁻¹) light conditions. B, Hypocotyl of aha2-4;mCitAHA2 seedling grown for 10 days under the bright light. C, Hypocotyl of aha2-4;mCitAHA2 seedling grown for 10 days under the dim light. D, Root of aha2-4;mCitAHA2 seedling grown for 10 days under the bright light. E, Root of aha2-4;mCitAHA2 seedling grown for 10 days under the dim light. F, Root growth curve of aha2-4;mCitAHA2 seedlings grown under the dim light. n = 7. Data is shown with the mean plus/minus the standard error of the mean. G, Root aha2-4;mCitAHA2 seedling grown for four days under the dim light. H, Root of aha2-4;mCitAHA2 seedling grown for 10 days under the dim light. For B-E and G-H, images are a Maximum Intensity Projection (MIP) of fluorescent Confocal Laser Scanning Microscope (CLSM) optical sections. Scale bars indicate 50 microns for B-C and 20 microns for D-E and G-H. I, Quantitation of mCitAHA2 fluorescence intensity. The mean fluorescent intensity values were calculated along root length by 21-pixel window size corresponding to ~8 microns from three 3D images of aha2-4;mCitAHA2 roots grown the dim or bright conditions (Supplemental Figure S2). Top, Heat map for aha2-4;mCitAHA2 intensity scale; white indicates the highest intensity and black indicates the lowest intensity. Middle, Images of aha2-4;mCitAHA2 seedling roots grown under the dim or bright light conditions. The x-axis of the root images corresponds to the scale shown in the bottom chart showing the intensity profiling. Bottom, Quantified intensity profiles for aha2-4;mCitAHA2 seedling roots grown under the dim or bright light conditions. Approximately 60 optical slices were summed at a given pixel position. Voxels were categorized into two groups: high intensity (yellow) or medium intensity (red). Subtracting the number of low intensity voxels from the high intensity voxels produced accumulation index (shown with blue plot).

Figure 3. Visualization and quantitation of intracellular localization of mCitAHA2 fluorescent signals in aha2-4;mCitAHA2 roots grown under dim light for 11 days. A, Quantitation of intracellularly localized mCitAHA2 structures (i.e., blobs). Root region 212 μm from the tip in the 3D images were segmented into meristem (61.28 μm from the tip), transition zone (63.77 μm above the meristem), and elongation zone (86.95 μm above the transition zone). For each region, surface rendering and identification of intracellularly localized structures was performed as detailed in Materials and Methods (an example of the surfaces generated is shown in Supplemental Fig. S5). Graph shows mean number of intracellular structures ± standard deviation (n=3). B, Three dimensional image of root tip of aha2-4;mCitAHA2 seedling grown for 10 days under the dim light. Image was detected by mCitrine emission. Bar, 20 μm. C,
Propidium iodide (PI) staining of the identical aha2-4;mCitAHA2 seedling shown in B. Image was detected by PI emission. D. Two-channel merge of B and C. Regression values for co-localization of mCitAHA2 and PI were shown at the right of the corresponding regions. E, Magnified view of the transition zone that was visualized in the mCitAHA2 channel shown in B. Bar, 10 μm. F. Magnified view of PI-stained cells in the same region shown in E. G, Orthogonal view of mCitAHA2 root. Top, XZ section of the root at the position indicated with green line shown in the bottom image. Bottom, XY section of the root at the position indicated with blue line shown in the top image. Bar, 15 μm. H, Magnified view of the root regions showing the transition of mCitAHA2 localization from the intracellular compartments to the plasma membrane. Bar, 15 μm. I, Diagram showing the interpretation of the fluorescent raw image shown in H. To assist the interpretation of changes in mCitAHA2 localization, the image was reproduced by drawing. J, Annotation of cell types and mCitAHA2 cellular localization.

Figure 4. Measurement of root surface pH. A, Image of aha2-4;mCitAHA2 root grown under dim light for 10 days. A, Root image was visualized with mCitrine channel detection. The yellow color in the bathing media is due to overlapping emission from FITC dextran. A root was submerged in a unbuffered solution containing 1 mM KCl, 1mM CaCl₂, and 3 mg/mL Fluorescein Dextran. Bar, 20 μm. B, Visualization of FITC-dextran as a pH probe for measuring the root surface pH of the identical plant. Brighter green indicates higher pH values. C, Intensity profiling the root surface pH. Left, Color scale for pH. Right, heat map of pH values at the root surface. Region surrounded by white line was used to measure root surface pH. D, Quantitation of the mean pH values at the root surface. Data is shown as the mean +/- standard error of the mean. n=4. E, Image of aha2-4;mCitAHA2 root grown under dim light for 10 days and visualized with mCitrine channel detection. A root was submerged in a buffered solution containing 10 mM MES (pH5.5), 1 mM KCl, 1 mM CaCl₂, and 3 mg/mL FITC dextran. F, Visualization of FITC-dextran as a pH probe for measuring the root surface pH. G, Profiling the root surface pH. Left, Color scale for pH. Right, heat map of pH values at the root surface. H, Quantitation of pH values at the root surface. Data is shown as the mean +/- standard error of the mean. n=4.

Figure 5. FERONIA-dependent root growth suppression and mCitAHA2 intracellular accumulation. A, Effect of fer-4 mutation on dim-light dependent root growth suppression. The aha2-4;mCitAHA2 or fer-4; aha2-4;mCitAHA2 seedlings were grown under various light intensity conditions for 10 days. n=7. Data is shown as the mean plus/minus standard deviation. B, Images of aha2-4;mCitAHA2 and fer-4; aha2-4;mCitAHA2 seedlings grown under the dim light for 10 days. Bar, 1 cm. C, Images of aha2-4;mCitAHA2 and fer-4; aha2-4;mCitAHA2 seedlings grown under the bright light for 10 days. D, Fluorescent intensity profiling of aha2-4;mCitAHA2 root grown under the dim light for 10 days. E, Fluorescent intensity profiling of aha2-4;mCitAHA2 root grown under the bright light for 10 days. F, Fluorescent intensity profiling of fer-4;aha2-4;mCitAHA2 root grown under the dim light for 10 days. G, Fluorescent intensity profiling of fer-4;aha2-4;mCitAHA2 root grown under the bright light for 10 days. Scale bar indicates 20 μm for D-G. H, Comparison of mCitAHA2 protein abundance between FER wildtype and fer-4 knockout plants grown under bright or dim light conditions. Crude protein extracts (6.5 μg) from seedlings grown for 10 days under bright or dim light were analyzed. Top, Immuno blot detected with GFP antibody. Bottom, Ponceau S staining of the identical blot is shown as a loading control. I, Quantitation of GFP positive signals shows that
fer-4;aha2-4;mCitAHA2 plants grown under dim light contain more mCitAHA2 protein than aha2-4;mCitAHA2 plants. n=3. Data is shown as the mean plus/minus standard errors.

LITERATURE CITED


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Figure 1. Expression of fluorescently labeled AHA2 in plants. A, Membrane topology of mCitrineAHA2 (mCitAHA2) fusion protein. The mCitrine fluorescent protein sequence was inserted at the amino acid position Leu4 located in the cytoplasmic face of AHA2 sequence. B, Root growth measurements of mCitAHA2 expressing plants. Arabidopsis seedlings of wildtype, aha2-4 mutant, aha2-4;AHA2 (wildtype complementation) or aha2-4;mCitAHA2 were grown for three days onto 1/2MS media, then transferred to the media supplemented with 100 mM KCl, and grown for additional 10 days. C, Immunological detection of mCitAHA2 protein from aha2-4;mCitAHA2 plants. Ten-day-old seedlings were extracted to enrich the plasma membrane fraction using the two-phase partition method. Sol., fraction containing soluble proteins. LP, lower phase fraction containing endomembrane proteins. UP, upper phase fraction containing plasma membrane proteins. Fifteen microgram of proteins were loaded onto protein gel. Blot was probed with anti-GFP (Right) and subsequently stained with Ponceau S to visualize proteins (Left).
Figure 2. Organ length and mCitAHA2 localization in aha2-4;mCitAHA2 plants grown under bright or dim light conditions.

A, Hypocotyl and root length of aha2-4;mCitAHA2 seedlings grown for 10 days under dim (2.2 umol/m2/sec) or bright (10 umol/m2/sec) light conditions. B, Hypocotyl of aha2-4;mCitAHA2 seedling grown for 10 days under the bright light. C, Hypocotyl of aha2-4;mCitAHA2 seedling grown for 10 days under the dim light. D, Root of aha2-4;mCitAHA2 seedling grown for 10 days under the bright light. E, Root of aha2-4;mCitAHA2 seedling grown for 10 days under the dim light. F, Root growth curve of aha2-4;mCitAHA2 seedlings grown under the dim light. n = 7. Data is shown with the mean plus/minus the standard error of the mean. G, Root aha2-4;mCitAHA2 seedling grown for four days under the dim light. H, Root of aha2-4;mCitAHA2 seedling grown for 10 days under the dim light. For B-E and G-H, images are a Maximum Intensity Projection (MIP) of fluorescent Confocal Laser Scanning Microscope (CLSM) optical sections. Scale bars indicate 50 microns for B-C and 20 microns for D-E and G-H. I, Quantitation of mCitAHA2 fluorescence intensity. The mean fluorescent intensity values were calculated along root length by 21-pixel window size corresponding to ~8 microns from three 3D images of aha2-4;mCitAHA2 roots grown the dim or bright conditions (Supplemental Figure S2). Top, Heat map for aha2-4;mCitAHA2 intensity scale; white indicates the highest intensity and black indicates the lowest intensity. Middle, Images of aha2-4;mCitAHA2 seedling roots grown under the dim or bright light conditions. The x-axis of the root images corresponds to the scale shown in the bottom chart showing the intensity profiling. Bottom, Quantified intensity profiles for aha2-4;mCitAHA2 seedling roots grown under the dim or bright light conditions. Approximately 60 optical slices were summed at a given pixel position. Voxels were categorized into two groups: high intensity (yellow) or medium intensity (red). Subtracting the number of low intensity voxels from the high intensity voxels produced accumulation index (shown with blue plot).
Figure 3. Visualization and quantitation of intracellular localization of mCitAHA2 fluorescent signals in aha2-4;mCitAHA2 roots grown under dim light for 11 days. A, Quantitation of intracellularly localized mCitAHA2 structures (i.e., blobs). Root region 212 μm from the tip in the 3D images were segmented into meristem (61.28 μm from the tip), transition zone (63.77 μm above the meristem), and elongation zone (86.95 μm above the transition zone). For each region, surface rendering and identification of intracellularly localized structures was performed as detailed in Materials and Methods (an example of the surfaces generated is shown in Supplemental Fig. S5). Graph shows mean number of intracellular structures ± standard deviation (n=3). B, Three-dimensional image of root tip of aha2-4;mCitAHA2 seedling grown for 10 days under the dim light. Image was detected by mCitrine emission. Bar, 20 um. C, Propidium iodide (PI) staining of the identical aha2-4;mCitAHA2 seedling shown in B. Image was detected by PI emission. C, Two-channel merge of B and C. Regression values for co-localization of mCitAHA2 and PI were shown at the right of the corresponding regions. E, Magnified view of the transition zone that was visualized in the mCitAHA2 channel shown in B. Bar, 10 um. F, Magnified view of PI-stained cells in the same region shown in E. G, Orthogonal view of mCitAHA2 root. Top, XZ section of the root at the position indicated with green line shown in the bottom image. Bottom, XY section of the root at the position indicated with blue line shown in the top image. Bar, 15 um. H, Magnified view of the root regions showing the transition of mCitAHA2 localization from the intracellular compartments to the plasma membrane. Bar, 15 um. I, Diagram showing the interpretation of the fluorescent raw image shown in H. To assist the interpretation of changes in mCitAHA2 localization, the image was reproduced by drawing. J, Annotation of cell types and mCitAHA2 cellular localization.
Figure 4. Measurement of cell-surface pH. A, Image of aha2-4;mCitAHA2 root grown under dim light for 10 days. Root image was visualized with mCitrine channel detection. The yellow color in the bathing media is due to overlapping emission from FITC dextran. A root was submerged in a unbuffered solution containing 1 mM KCl, 1 mM CaCl₂, and 3 mg/mL Fluorescein Dextran. Bar, 20 μm. B, Visualization of FITC-dextran as a pH probe for measuring the root surface pH of the identical plant. Brighter green indicates higher pH values. C, Intensity profiling the root surface pH. Left, Color scale for pH. Right, heat map of pH values at the root surface. Region surrounded by white line was used to measure root surface pH. D, Quantitation of the mean pH values at the root surface. Data is shown as the mean +/- standard error of the mean. n=4. E, Image of aha2-4;mCitAHA2 root grown under dim light for 10 days and visualized with mCitrine channel detection. A root was submerged in a buffered solution containing 10 mM MES (pH 5.5), 1 mM KCl, 1 mM CaCl₂, and 3 mg/mL FITC dextran. F, Visualization of FITC-dextran as a pH probe for measuring the root surface pH. G, Profiling the root surface pH. Left, Color scale for pH. Right, heat map of pH values at the root surface. H, Quantitation of pH values at the root surface. Data is shown as the mean +/- standard error of the mean. n=4.
Figure 5. FERONIA-dependent root growth suppression and mCitAHA2 intracellular accumulation. A, Effect of fer-4 mutation on dim-light dependent root growth suppression. The aha2-4; mCitAHA2 or fer-4; aha2-4; mCitAHA2 seedlings were grown under various light intensity conditions for 10 days. n=7. Data is shown as the mean plus/minus standard deviation. B, Images of aha2-4; mCitAHA2 and fer-4; aha2-4; mCitAHA2 seedlings grown under the dim light for 10 days. Bar, 1 cm. C, Images of aha2-4; mCitAHA2 and fer-4; aha2-4; mCitAHA2 seedlings grown under the bright light for 10 days. D, Fluorescent intensity profiling of aha2-4; mCitAHA2 root grown under the dim light for 10 days. E, Fluorescent intensity profiling of fer-4; aha2-4; mCitAHA2 root grown under the bright light for 10 days. F, Fluorescent intensity profiling of aha2-4; mCitAHA2 root grown under the dim light for 10 days. G, Fluorescent intensity profiling of fer-4; aha2-4; mCitAHA2 root grown under the bright light for 10 days. Scale bar indicate 20 micron for D-G.

H, Comparison of mCitAHA2 protein abundance between FER wildtype and fer-4 knockout plants grown under bright or dim light conditions. Crude protein extracts (6.5 ug) from seedlings grown for 10 days under bright or dim light were analyzed. Top, Immuno blot detected with GFP antibody. Bottom, Ponceau S staining of the identical blot is shown as a loading control. I, Quantitation of GFP positive signals shows that fer-4; aha2-4; mCitAHA2 plants grown under dim light contain more mCitAHA2 protein than aha2-4; mCitAHA2 plants. n=3. Data is shown as the mean plus/minus standard errors.


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