Clathrin Heavy Chain Subunits Coordinate Endo- and Exocytic Traffic and Affect Stomatal Movement

Emily R. Larson,a,2 Eva Van Zelm,a,b Camille Roux,c Annie Marion-Poll,c and Michael R. Blatt,a

aLaboratory of Plant Physiology and Biophysics, University of Glasgow, Glasgow G12 8QO, United Kingdom
bUniversity of Amsterdam, Faculty of Science, Graduate School of Life and Earth Sciences, 1090 GE Amsterdam, The Netherlands
cInstitut Jean-Pierre Bourgin, Institut National de la Recherche Agronomique, AgroParisTech, Centre National de la Recherche Scientifique, Université Paris-Saclay, 78000 Versailles, France

The current model for vesicular traffic to and from the plasma membrane is accepted, but the molecular requirements for this coordination are not well defined. We have identified the hot ABA-deficiency suppressor1 mutant, which has a stomatal function defect, as a clathrin heavy chain1 (CHC1) mutant allele and show that it has a decreased rate of endocytosis and growth defects that are shared with other chc1 mutant alleles. We used chc1 alleles and the related chc2 mutant as tools to investigate the effects that clathrin defects have on secretion pathways and plant growth. We show that secretion and endocytosis at the plasma membrane are sensitive to CHC1 and CHC2 function in seedling roots and that chc mutants have physiological defects in stomatal function and plant growth that have not been described previously. These findings suggest that clathrin supports specific functions in multiple cell types. Stomata movement and gas exchange are altered in chc mutants, indicating that clathrin is important for stomatal regulation. The aberrant function of chc mutant stomata is consistent with the growth phenotypes observed under different water and light conditions, which also are similar to those of the secretory SNARE mutant, sup121. The sup121 and chc mutants have impaired endocytosis and exocytosis compared with the wild type, indicating a link between SYPI21-dependent secretion and clathrin-dependent endocytosis at the plasma membrane. Our findings provide evidence that clathrin and SYPI21 functions are important for the coordination of endocytosis and exocytosis and have an impact on stomatal function, gas exchange, and vegetative growth in Arabidopsis (Arabidopsis thaliana).

Vesicle traffic to and from the plasma membrane plays an integral role in regulating protein localization and activity, membrane composition, and cell surface area. This traffic contributes to cell growth and development and to a wide range of physiological processes that enable plants to survive under varying environmental conditions. Exocytosis facilitates the distribution of proteins such as ion channels, receptors, and transporters to the cell surface at specific times and regions of the plasma membrane; it provides a mechanism for delivering cell wall components to the apoplast and supports cell growth and resistance to pathogens (Baluška et al., 2002; Sutter et al., 2007; Tyrrell et al., 2007; Bassham and Blatt, 2008). Vesicle fusion adds a new lipid bilayer to the plasma membrane that accommodates changes in cell shape and cell size (Battey et al., 1999; Geelen et al., 2002; Grefen et al., 2011). These fusion events are mediated by Soluble NSF Attachment Protein Receptor (SNARE) complexes that assemble from subunits present at both the plasma membrane and the surface of the docking vesicles (Rothman and Wieland, 1996; Sanderfoot et al., 2000; Grefen and Blatt, 2008). SNARE proteins are well conserved in eukaryotes, although in plants this family of proteins is greatly expanded, suggesting that vesicle traffic is either more complex or more abundant in comparison with the animal kingdom (Uemura et al., 2004; Sanderfoot, 2007; Honsbein et al., 2011).

Exocytic pathways to the plasma membrane are complemented by endocytic traffic that removes and recycles proteins and lipids from the plasma membrane, thereby regulating their presence and activity (Becker et al., 2003; Dhonukshe et al., 2007; Sutter et al., 2007; Kitakura et al., 2011). This other side of the traffic cycle at the plasma membrane is equally critical for physiology and development. The polarized localization of PIN proteins, for example, is maintained by the coordinated transport between the plasma membrane...
and endomembrane compartments to provide directional gradients of auxin in plants (Feraru and Friml, 2008; Kitakura et al., 2011; Jäskil et al., 2013, 2016). When endocytosis is disrupted, this polarized expression is lost and auxin gradients dissipate (Kitakura et al., 2011). SNARE proteins localized on vesicles also must be returned to their originating, internal membranes after vesicle fusion, which requires endocytosis (Abeliovich et al., 1999; Enami et al., 2009). Indeed, although direct evidence is scant, there is a general agreement that both exocytic and endocytic pathways of the trafficking cycle at the plasma membrane are important for multiple plant cell functions and that the two processes must be tightly coordinated. Certainly, suppressing secretory traffic is known to have an impact on endocytosis (Battey et al., 1999; Geldner, 2004; Bandmann et al., 2011), but whether altered endocytosis affects exocytic traffic in many instances remains an open question.

Clathrin is a key structural protein that forms a lattice-like complex that includes two heavy chain subunits (CHC1 and CHC2) and two light chain subunits (CLC1 and CLC2). The subunits assemble a cage-like scaffold around developing vesicles to support vesicle formation at the plasma membrane and endosomal membranes in plants and animals (Goud et al., 1991; Blackbourough and Jackson, 1996; Puertollano et al., 2001; Holstein, 2002; Baisa et al., 2013). This scaffolding dissociates once the vesicle is fully formed and detached from the initial membrane by dynamin (Damke et al., 1994; Konopka et al., 2008), allowing the now naked vesicle to traffic to its destination. In plants, although a reduction in endocytosis has been noted in the roots of chc2 mutants, the known single chc mutant alleles do not present severe phenotypes (Kitakura et al., 2011), suggesting a redundancy in function of the subunits. However, a tamoxifen-inducible dominant negative mutant line (HUB1) allows for the observation of the severe repercussions of the total loss of clathrin function. When induced, the dominant negative HUB1 mutant has stunted growth in multiple organs, indicating a requirement for clathrin function for the development of multiple cell types (Liu et al., 1995, 1998; Tahara et al., 2007; Robert et al., 2010). The endocytic and exocytic machinery are widely expressed in all cell types, and their functions, while similar, support the specialization of the different cell types that make up the organism. Therefore, all cells require both secretion and endocytosis for their growth and function. Clathrin is a major player in the regulation of endocytosis at the plasma membrane, but how its function relates to the coordination of vesicle traffic in plant cells is less defined.

Stomatal guard cells provide a particularly good example of the juxtaposition of exocytic and endocytic traffic in plants and our limited understanding of their coordination. Guard cells control transpiration by regulating the aperture of the stomatal pores in the leaf epidermis to facilitate gas exchange for photosynthesis while minimizing transpirational water loss. Guard cells undergo a 2- to 3-fold increase in cell volume and a 30% to 40% increase in surface area as the stoma opens, and these processes are reversed as the guard cells close the stomatal pore (Blatt, 2000; Shope et al., 2003; Meckel et al., 2007; Bauer et al., 2013). Changes in cell volume are driven by solute uptake and loss, primarily of K+ salts, and are regulated by a number of signals including changes in cytosol-free [Ca2+] (Blatt, 2000; Minguet-Parramon et al., 2016). Membrane traffic, including the traffic of K+ channels, plays a key part in these events (Sutter et al., 2007; Eisenach et al., 2012). Furthermore, the block of exocytosis has been shown to suppress stomatal closure (Leyman et al., 1999), even though closure is commonly associated with endocytosis and the internalization of membrane material and proteins (Eisenach et al., 2012). In short, while all evidence supports a requirement for vesicle traffic in stomatal function, it is not clear how the two sides of the trafficking cycle to and from the guard cell plasma membrane are linked.

We asked how the clathrin machinery contributes to the coordination of plasma membrane traffic and cell function using chc1 and chc2 mutants. We also further characterized the hot ABA-deficiency suppressor1 (has1) mutation as a new CHC1 mutant allele (Plessis et al., 2011), which has a premature stop codon in the CHC1 gene in Arabidopsis (Arabidopsis thaliana). While clathrin functions at several internal membranes, the effect clathrin has on traffic is obvious at the plasma membrane, which can be readily monitored with the use of FM dyes in expanding root epidermal cells. Stomata of the has1 mutant are more closed under dehydration stress conditions (Plessis et al., 2011), indicating a new phenotype associated with the CHC1 mutant alleles and providing evidence for clathrin-mediated endocytosis in stomatal function. We further characterize the phenotypes of the chc1 and chc2 mutants and show that they are defective in both endocytosis and exocytosis, which, in turn, affects cellular functions and environmental response. We also show that the secretary syp121 mutant has a reduced rate of endocytosis, again supporting a mutual requirement for both arms of the trafficking cycle for productive vesicle transport at the plasma membrane. We further suggest that SYP121 and clathrin contribute to the coordination of vesicle transport at the plasma membrane and that disruption of this coordination can be observed in the defective stomatal behavior in the syp121 and chc mutants, which inhibits these mutants from quickly responding to their changing environments. The downstream effects of these molecular defects result in altered plant growth.

RESULTS

The has1 Mutation Is a New chc1 Allele in Arabidopsis

The has1 mutant was originally identified in an infrared imaging screen of a γ-ray mutagenized population of the ABA deficient3 (aba3-1) mutant. The has1 aba3-1 double mutation suppressed the cold-leaf phenotype associated with increased transpiration in the original aba3-1 mutant. Fine-mapping localized the has1 mutation

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to a region on the lower arm of chromosome 3 between 2.1 and 3.7 Mb (Plessis et al., 2011). After backcrossing to the wild type Columbia-0 (Col-0) and segregation of the aba3-1 mutation, the has1 mutant was shown to have defects in stomatal responses to abscisic acid (ABA), which conferred a water stress-tolerant phenotype (Plessis et al., 2011). With further mapping, we reduced the interval to 0.4 Mb, and next-generation DNA sequencing of has1 identified a five-nucleotide deletion in the 23rd exon of the CHC1 gene (At3g11130) that introduces a frame shift and produces a premature stop codon into the CHC1 gene (Supplemental Fig. S1). To confirm the has1 mutant as a chc1 mutant, we included other chc1 mutant alleles and those of a close family member, CHC2, to explore their effects on secretion and the connection between clathrin-mediated endocytosis and plasma membrane transport. When gene-specific primers (Kitakura et al., 2011) were used with genomic DNA from wild type Col-0 and chc1-1, chc1-2, chc1-3, chc2-1, and chc2-2 mutants, PCR products could be amplified only from the wild-type template, while the products amplified using the T-DNA left border-specific primer with gene-specific primers were observed only in the mutant lines (Supplemental Fig. S1B). These results indicate that gene transcription in the chc mutants is completely disrupted and that each of the chc mutant alleles is a null mutant. There is no effective antibody products and con

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amino acid sequence of endocytic activity.

and induced HUB1 seedlings, endocytosis was greatly re-

clathrin function was completely abolished in the in-

25% that of the wild-type control. Furthermore, when

clathrin function was completely abolished in the in-

induced HUB1 seedlings, endocytosis was greatly re-

exponential function:

\[ R_t = \frac{F_{int}}{F_{per}} = R_o + R_{max}(1-e^{-kt}) \] (1)

where \( R_t \) at time \( t \) is the ratio of fluorescence signals; \( F_{int} \) and \( F_{per} \) are the fluorescent signals measured inside the cell and at its perimeter, respectively; \( R_o \) is the initial fluorescence signal ratio or offset; \( R_{max} \) is the maximum amplitude of the fluorescence ratio; and \( k \) is the rate constant for internalization. Curve fittings were carried out jointly with data from the different lines while holding one or more of these parameters constant between data sets to minimize the number of free parameters. The best and visually satisfactory fittings were obtained when \( R_o \) and \( R_{max} \) were held constant and only \( k \) was allowed to vary between data sets. This analysis indicated that the rates of endocytosis were significantly slower in the has1, chc1-2, and chc2-1 mutant lines when compared with the wild type (Fig. 1B). These mutants each yielded an endocytic rate that was roughly 25% that of the wild-type control. Furthermore, when clathrin function was completely abolished in the induced HUB1 seedlings, endocytosis was greatly reduced to approximately 3% of wild-type endocytic rates (Table I). This analysis demonstrates the shared endocytic defect of has1 and chc mutants, consistent with has1 being an additional chc1 mutant allele. While it has already been shown that the chc2 mutant has reduced endocytosis (Kitakura et al., 2011), our data further illustrate that this also is observable in chc1 mutant alleles using our method and is due to a significant reduction in the rate of endocytosis rather than to a general loss of endocytic activity.

The chc1 and chc2 Mutants Have Reduced Rates of Endocytosis

We compared the rates of endocytosis in the wild type (Col-0), chc1, chc2, has1, and induced HUB1 dominant negative mutant seedlings grown in liquid culture by measuring the internalization of the styryl dye FM4-64 over time in epidermal root cells using laser scanning confocal microscopy. The HUB1 lines express a truncated form of CHC1 under the control of a tamoxifen-inducible promoter. When the HUB1 fragment is expressed, it binds together with endogenous clathrin subunits to form a nonfunctional lattice that cannot produce a vesicle coat (Liu et al., 1995). Thus, expressing the HUB1 fragment effectively blocks all clathrin-mediated endocytosis. To induce the expression of the HUB1 fragment, seedlings were incubated with tamoxifen for 2 d prior to the experiment, and induction was confirmed by the observation of multiple severe growth defects (Supplemental Fig. S2). Our observations showed a slower rate of FM4-64 internalization in the has1 and chc mutants compared with the wild type and the uninduced HUB1 line; the induced HUB1 line had almost no internalization of FM4-64 over the course of 30 min (Fig. 1). To quantify these results, we fitted the internal FM4-64 fluorescence signals to an exponential function to extract the time constants for its internalization. We approximated internalization using a single exponential function:

\[ R_t = \frac{F_{int}}{F_{per}} = R_o + R_{max}(1-e^{-kt}) \] (1)

where \( R_t \) at time \( t \) is the ratio of fluorescence signals; \( F_{int} \) and \( F_{per} \) are the fluorescent signals measured inside the cell and at its perimeter, respectively; \( R_o \) is the initial fluorescence signal ratio or offset; \( R_{max} \) is the maximum amplitude of the fluorescence ratio; and \( k \) is the rate constant for internalization. Curve fittings were carried out jointly with data from the different lines while holding one or more of these parameters constant between data sets to minimize the number of free parameters. The best and visually satisfactory fittings were obtained when \( R_o \) and \( R_{max} \) were held constant and only \( k \) was allowed to vary between data sets. This analysis indicated that the rates of endocytosis were significantly slower in the has1, chc1-2, and chc2-1 mutant lines when compared with the wild type (Fig. 1B). These mutants each yielded an endocytic rate that was roughly 25% that of the wild-type control. Furthermore, when clathrin function was completely abolished in the induced HUB1 seedlings, endocytosis was greatly reduced to approximately 3% of wild-type endocytic rates (Table I). This analysis demonstrates the shared endocytic defect of has1 and chc mutants, consistent with has1 being an additional chc1 mutant allele. While it has already been shown that the chc2 mutant has reduced endocytosis (Kitakura et al., 2011), our data further illustrate that this also is observable in chc1 mutant alleles using our method and is due to a significant reduction in the rate of endocytosis rather than to a general loss of endocytic activity.

Secretion Is Inhibited by Defects in Clathrin-Mediated Endocytic Pathways

Given that the trafficking pathways to and from the plasma membrane are considered to rely on each other (Murphy et al., 2005; Tanaka et al., 2014), we asked if secretion was altered in the chc mutants using a transient expression assay in seedling roots. To visualize secretion in the chc mutants, seedling roots were transiently transformed with a single, multicistronic vector that allows for the expression of a secreted YFP (secYFP) protein, a retained GFP-HDEL protein, and a gene of interest. This vector enables the quantification of secretion using GFP-HDEL as a reference for ratio-metric analysis and as a control for transformation (Karnik et al., 2013). Seedlings were transiently transformed with either the vector carrying secYFP and GFP-HDEL alone to visualize bulk secretion patterns in

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the different genetic backgrounds or with the cytoplasmic fragment of the plasma membrane SNARE protein, SYP121 (SYP121<sup>DC</sup>), expressed in the third cassette as a positive control for secretion blockage (Karnik et al., 2013). When secretion occurs without blockage in transformed root epidermal cells, YFP fluorescence is lost as the secYFP protein diffuses out into the apoplast, leading to a low ratio of secYFP/GFP-HDEL fluorescence in the tissue. However, in the presence of secretory antagonists such as SYP121<sup>DC</sup>, the secYFP is trapped within the cells and the secYFP/GFP ratio increases (Grefen et al., 2015). When the secYFP/GFP-HDEL ratios were compared between the different genotypes, wild-type roots expressing SYP121<sup>DC</sup> elicited a higher level of internal secYFP signal than when expressing secYFP and GFP-HDEL alone; however, the two independent chc1 mutant alleles had a block in secretion regardless of the presence of SYP121<sup>DC</sup> (Fig. 2). These data indicate that clathrin function affects secretion pathways to the plasma membrane as well as reduces the rate of endocytosis from the plasma membrane.

The reduction in both endocytosis and secretion in the chc mutants prompted us to ask if endocytosis is altered in a known secretory mutant. The Qa-SNARE, SYP121, is required for secretory pathways to the plasma membrane (Fasshauer et al., 1998; Geelen et al., 2002; Geldner, 2004; Enami et al., 2009), shown by a significant block of secretion in the syp121 null mutant (Eisenach et al., 2012). We hypothesized that if secretion is altered by defects in clathrin endocytic machinery function, then endocytosis could be altered when secretory machinery function is compromised. We generated new FM4-64 internalization measurements to compare endocytic rates in Col-0 and syp121, syp122, chc1-2, and chc2-1 mutant lines and calculated the half-time for each genotype. The internalization half-time of the FM6-64 dye in syp121 mutants was greater than that in the wild type, indicating that endocytosis is reduced in the syp121 secretory mutant as well as in the endocytic chc mutants compared with the wild type (Fig. 3; Table II). Endocytosis in the less severe secretion mutant syp122 was not significantly affected. These data support a model for the link between the functions of molecular machinery that mediate both inward and outward patterns of vesicle transport.

**Figure 1.** The rate of endocytosis is reduced in the chc mutant lines. A, Seedling roots were labeled with FM4-64 to measure internalization over time. The endocytosis rate was reduced in has1 and other chc1 alleles as well as in the chc2 mutant compared with the wild type (Col-0) and the untreated HUB1 line (−tam). Almost no internalization is observed in the HUB1 line pretreated with tamoxifen (+tam). The white frames in the Col-0 images highlight the boundary between the periphery (F<sub>per</sub>) and internal spaces (F<sub>int</sub>) of single cells that were used for fluorescence measurement. Bars = 10 μm. B, Graphical representation of FM4-64 internalization in Col-0, chc mutant lines, and the inducible dominant negative mutant HUB1 line treated with (+) and without (−) tamoxifen over time. Curves are joint fittings using Equation 1 with R<sub>o</sub> and R<sub>max</sub> held in common between data sets; the y axis is plotted as the ratio of fluorescence values (F<sub>int</sub>/F<sub>per</sub>) in reference to Equation 1. The has1 and chc mutants show approximately 75% reduction in the rate of endocytosis, while endocytosis in the induced HUB1 seedlings was reduced to approximately 3% of the wild-type control. Values are means ± se; n > 30 cells per genotype.
The chc1 Mutation Affects Stomatal Function

The stomatal defect reported in the has1 mutant (Plessis et al., 2011) inspired us to investigate the stomatal activity in the other chc mutants in more detail. For these experiments, we applied exogenous Ca\(^{2+}\) to mimic ABA-triggered stomatal closure (Allen et al., 2001, 2002; Yang et al., 2006; Cho et al., 2009; Eisenach et al., 2012). We again used the HUB1 lines as an additional control for the chc1 and has1 mutant alleles. Epidermal peels were mounted in a custom perfusion chamber and incubated in a depolarizing buffer (DB; 0 mM Ca\(^{2+}\) and 60 mM K\(^{+}\)) to stimulate stomatal opening and then replaced with a hyperpolarizing buffer (HB; 6 mM Ca\(^{2+}\) and 0.1 mM K\(^{+}\)) to stimulate stomatal closing (Allen et al., 2001; Eisenach et al., 2012). The wild-type stomata closed in response to the HB and reopened with DB treatment over the 3 h of treatment (Fig. 4). The has1 mutant stomata closed and reopened slower under these treatment conditions over the measured time period. After induction, the HUB1 line was unable to reopen after HB-induced stomatal closure. These data are similar to those seen in plants treated with the endosomal recycling inhibitor brefeldin A (BFA; Geldner et al., 2001; Nebenführ et al., 2002; Eisenach et al., 2012) and underscore the requirement for both endocytic and exocytic pathways in stomatal function, for which clathrin is required. We also measured the transpiration in whole plants to assess the stomatal dynamics of another chc1 mutant allele in response to changing CO\(_2\) levels (Fig. 4C). The results were consistent with the previous stomatal aperture measurements in has1 and identify both a slower closing and reopening of stomata, which could contribute to the overall chc1 mutant phenotype.

We hypothesized that the endocytosis defects observed in the roots of chc1 mutants could represent a similar defect in stomata and contribute to their defective behavior. We infused leaves of Arabidopsis wild-type and mutant lines with FM4-64 and measured its internalization over time in guard cells using a similar methodology to that described for root epidermal cells. While FM dyes are reported to label endosomal compartments in guard cells of Vicia faba (Meckel et al., 2004), quantitative measurement of FM dye uptake for endocytic analysis in Arabidopsis guard cells is not frequently reported. We found that the vacuum infusion of fully expanded leaves provided the best images for assessing membrane and internal compartment labeling in Arabidopsis guard cells using a 40× objective under oil immersion. We again observed a significantly slower rate of internalization of FM4-64 in the chc1 guard cells compared with the wild type, indicating that the reduction in endocytosis in the chc1 mutants is not cell type specific and also correlates to the stomatal defect in the clathrin mutants (Fig. 4D; Table III).

### Table I. Comparison of FM4-64 endocytosis rates in wild-type and chc mutant root epidermal cells

Fitted rates for endocytosis are from the data of Figure 1B. Data were fitted jointly to Equation 1, with values for \(R_0\) and \(R_{\text{max}}\) held in common between data sets, yielding \(R_0\) of 0.134 ± 0.005 and \(R_{\text{max}}\) of 0.158 ± 0.006. Fittings to the individual mutant lines yielded statistically equivalent results.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Endocytosis Rate ((k))</th>
<th>(k) Relative to the Wild Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (Col-0), HUB –tam</td>
<td>0.118 ± 0.013</td>
<td>0.118 ± 0.013</td>
</tr>
<tr>
<td>has1, chc1-2, chc2-1</td>
<td>0.032 ± 0.003</td>
<td>0.27</td>
</tr>
<tr>
<td>HUB +tam</td>
<td>0.003 ± 0.002</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Figure 2. Secretion is inhibited in chc1 mutants. A. The expression of a multicistronic vector expressing secYFP, GFP-HDEL, and the cytoplasmic fragment of the SNARE protein SYP121 (SYP121\(^{DC}\)) inhibits secYFP secretion (top row) compared with the empty vector (bottom row). SYP121\(^{DC}\) blocks secretion in wild-type (Col-0) roots and enhances secretory blockage in the chc1 mutants. Bars = 50 \(\mu\)m. B. Graphical representation of the fluorescence data presented in A. Letters indicate statistical significance by ANOVA, where \(P = 0.005\) using the Holm-Sidak model. Values are means ± se; \(n = 6\) seedlings per genotype. Representative data from one of at least three independent experiments are shown.
Loss of Clathrin Function Results in Growth Defects in chc Mutant Alleles

The has1 mutant was initially identified in a thermomaging screen of a mutant population and has stomata that are more closed under water stress (Plessis et al., 2011), consistent with the stomatal phenotype we describe for chc1 mutants in this report (Fig. 4). Indeed, the has1, chc1, and chc2 mutant alleles showed reduced water loss under rapid dehydration conditions when compared with wild-type plants (Fig. 5A). Thus, like the has1 mutant, other chc mutants are more tolerant to water loss than wild-type plants.

We asked whether the dehydration-tolerant phenotype of the chc mutants might translate to changes in plant growth during longer periods of water stress in soil. Wild-type and chc mutant plants were grown in well-watered conditions for 3 weeks before all watering was stopped for 15 d and plant appearance was monitored. To quantify these differences, the ratio of the fresh and dry weights was determined from rosettes sampled from each line and used as an index for water loss. In plants subjected to dehydration, we expected the fresh weight-to-dry weight ratios to decrease; these ratios were significantly lower in the chc1 and chc2 mutants than in wild-type plants (Fig. 5B). These measurements are consistent with the visual appearance of chc1 and chc2 mutants, which had less visible wilting after 9 d of dehydration (Fig. 5C).

We also examined dehydration tolerance in two dominant negative HUB1 lines (Liu et al., 1995) to confirm the clathrin-dependent effect. This construct has been expressed successfully in plants to investigate the requirement for clathrin-mediated endocytosis in cell function (Tahara et al., 2007; Robert et al., 2010; Kitakura et al., 2011). For our experiments, all genotypes were treated with 2 μM tamoxifen for 2 d before watering was stopped, and parallel experiments without tamoxifen treatment were conducted as a control. When induced with tamoxifen, two independent HUB1 lines presented a dehydration-tolerant phenotype when compared to the wild type (Supplemental Fig. S3). All of the dehydration stress results are consistent with the initial has1 phenotype (Plessis et al., 2011) and show that the chc mutants lose less water over time compared to the wild-type plants. The HUB1 mutant further showed that near-complete inhibition of clathrin function provides a dehydration-tolerant phenotype and that clathrin function impacts water stress responses in plants.

The reduced vesicle transport, stomatal movement defects, and dehydration tolerance in chc mutants suggest that mutant plant growth could be affected under conditions that require tight stomatal regulation. Following germination, cohorts of wild-type, has1, chc1, and chc2 plants were grown under combinations of 70 or 150 μmol m−2 s−1 light and 60% or 100% relative humidity (RH) in a short-day light regime for 2 weeks. When the mean rosette diameters were compared, the mutants were in most cases smaller than wild-type plants (Fig. 6). Under the control growth chamber settings (70 μmol m−2 s−1 light and 60% RH), the rosette diameters of the chc mutants were not significantly different from each other but were smaller than those of the wild type. No difference was observed between genotypes when plants were grown at elevated humidity (70 μmol m−2 s−1 light and 100% RH), suggesting that removing the pressure for water conservation masks the clathrin mutant growth phenotype. Indeed, when plants were grown in high light and reduced humidity conditions (150 μmol m−2 s−1 light and 60% RH), the chc mutants had significantly smaller rosette diameters compared to the wild type (Fig. 6).

Table II. Comparison of FM4-64 endocytosis in wild-type and syp121 and syp122 root epidermal cells

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Endocytosis Rate (k)</th>
<th>k Relative to the Wild Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (Col-0)</td>
<td>0.066 ± 0.009</td>
<td></td>
</tr>
<tr>
<td>syp121</td>
<td>0.014 ± 0.002</td>
<td>0.21</td>
</tr>
<tr>
<td>syp122</td>
<td>0.043 ± 0.005</td>
<td>0.65</td>
</tr>
<tr>
<td>chc1-2</td>
<td>0.012 ± 0.002</td>
<td>0.18</td>
</tr>
<tr>
<td>chc2-1</td>
<td>0.011 ± 0.002</td>
<td>0.16</td>
</tr>
</tbody>
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The reduced vesicle transport, stomatal movement defects, and dehydration tolerance in chc mutants suggest that mutant plant growth could be affected under conditions that require tight stomatal regulation. Following germination, cohorts of wild-type, has1, chc1, and chc2 plants were grown under combinations of 70 or 150 μmol m−2 s−1 light and 60% or 100% relative humidity (RH) in a short-day light regime for 2 weeks. When the mean rosette diameters were compared, the mutants were in most cases smaller than wild-type plants (Fig. 6). Under the control growth chamber settings (70 μmol m−2 s−1 light and 60% RH), the rosette diameters of the chc mutants were not significantly different from each other but were smaller than those of the wild type. No difference was observed between genotypes when plants were grown at elevated humidity (70 μmol m−2 s−1 light and 100% RH), suggesting that removing the pressure for water conservation masks the clathrin mutant growth phenotype. Indeed, when plants were grown in high light and reduced humidity conditions (150 μmol m−2 s−1 light and 60% RH), the chc mutants had significantly smaller rosette diameters compared to the wild type (Fig. 6).

The data for FM4-64 internalization into wild-type and chc mutant seedling roots is presented in Fig. 3. The half-times of FM4-64 internalization in the syp and chc mutants are greater than that measured in wild-type (Col-0) seedling roots. Letters indicate significant differences between genotypes from one of at least three independent experiments (ANOVA and the Holm-Sidak model, where P = 0.001; n = 30 cells measured across three seedlings per genotype.)
RH), which impose a requirement for water conservation in addition to high photosynthetic activity, the mutant plants were significantly smaller than wild-type plants. Interestingly, the chc2 mutants also were significantly smaller than the chc1 mutants under these conditions, suggesting a potential divergence in CHC1 and CHC2 functions. We measured steady-state transpiration in wild-type, chc1, and chc2 plants and observed lower transpiration levels in the clathrin mutant lines (Fig. 6C), which is consistent with stomata that are, in general, more often closed. Altogether, these data underscore the requirement for clathrin function during plant growth under conditions that increase photosynthetic activity and require well-regulated stomatal movements.

DISCUSSION

The impact of secretory inhibition on cell function is well documented, and several reports show that prolonged inhibition of secretory pathways ultimately affects endocytosis (Geldner et al., 2001; Nebenführ et al., 2002; Richter et al., 2007). These reports, however, are based mainly on studies with inhibitors like BFA that broadly affect traffic through the Golgi and the trans-Golgi network, which serves as a hub for both endocytic and exocytic trafficking pathways in plants. Thus, a clear delineation and assessment of the direct effects of one pathway alone are difficult to elucidate. Using genetic mutants allowed us to observe the effects of a direct loss of clathrin mechanical function during vesicle transport. With the genetic and physiological tools

**Table III. Comparison of FM4-64 endocytosis rates in wild-type and chc1 mutant stomata**

Internalization rates for endocytosis are from the data of Figure 4D. Analysis is shown for data ± se from at least five to eight stomata per genotype using a single exponential function (SigmaPlot version 11.0).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Endocytosis Rate (k)</th>
<th>k Relative to the Wild Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (Col-0)</td>
<td>0.24 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>chc1-2</td>
<td>6.3 × 10^{-5} ± 0.04</td>
<td>0.26 × 10^{-1}</td>
</tr>
</tbody>
</table>
available in plants, we can now show (1) that disruption of either exocytic or endocytic pathways in stomata as well as other cell types leads to physiological consequences, evident in the suppressed growth and enhanced dehydration resistance of the chc mutants; (2) that endocytosis and exocytosis are essential for stomatal function; and (3) that, where secretion is impaired in the clathrin mutants, endocytosis is impaired in the syp121 mutant, indicating that these pathways are linked in their function and in their effects on cell function and plant physiology.

**Endocytosis and Exocytosis Are Linked Pathways That Can Mutually Affect Each Other**

While endocytosis was examined previously in chc1 and chc2 mutants (Kitakura et al., 2011), the rates of this traffic have not been measured directly in order to link the consequences of the reduction in endocytosis to a physiological behavior. We found that single mutations in CHC1 or CHC2 decrease the rate of endocytosis, increasing the half-time for FM4-64 internalization by roughly 4-fold compared with that of wild-type cells (Fig. 1). Kitakura et al. (2011) also used FM4-64 internalization to assay endocytosis in chc mutants and end-point measurements after 6 min, an approach that did not detect differences in endocytic rates that are evident with a multiple measurement time-course experiment. Thus, our endocytic rate analysis is consistent with their end-point results, improves the characterization of chc1 endocytic defects, and highlights the importance of a kinetic analysis of vesicle traffic in these circumstances. The results from the single chc1 and chc2 mutants indicate that a partial block in clathrin function has a negative impact on plant cell growth and regulation. This requirement for clathrin-dependent vesicle traffic for plant cell growth may not be surprising when considered with the role that clathrin plays in auxin efflux and plant development (Tahara et al., 2007; Wang et al., 2013; Yu et al., 2016).

Current models of the relationship between endocytosis and exocytosis are based primarily on experiments with BFA and suggest that an extended block of exocytosis leads to the accumulation of vesicles from endocytic pathways within so-called BFA compartments. The BFA sensitivity of these pathways is related to ARF-GEF protein inhibition and the consequences for the associated GTPases (Nebenführ et al., 2002; Geldner, 2004; Richter et al., 2007; Teh and Moore, 2007; Robinson et al., 2008). In both cases, these ARF-GEFs

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**Figure 5.** The chc mutants are more tolerant to water loss than wild-type Arabidopsis. A, The has1, chc1, and chc2 mutants are more tolerant to rapid dehydration compared with wild-type (Col-0) plants, indicated by a lower percentage of water loss. Values are means ± se; n = 4. B, Water loss is reduced under longer dehydration conditions in the chc mutants compared with wild-type plants, as shown by the mean fresh weight-to-dry weight ratios from 15-d-old rosettes. Values are means ± se; n = 10 plants for each genotype. Letters indicate significance calculated from ANOVA, where P < 0.005 (Holm-Sidak). C, Representative images of plants from each genotype after 0, 3, 6, and 9 d of no watering (n = 5). Results are from one of three independent experiments.
serve as regulators of trafficking pathways and associate with endosomal compartments that are integral to recycling patterns at the plasma membrane (Geldner, 2004); thus, they target the junctions between endocytic and exocytic processes. In contrast, we show here through direct mutation of the clathrin machinery that trafficking to the plasma membrane is impaired when the major regulator of endocytosis at the plasma membrane is compromised (Fig. 2). Furthermore, the stark phenotypic similarities between the syp121 and chc mutants are genetic indicators of a mutual functional dependency between endocytic and exocytic mechanisms (Figs. 2, 3, and 6). These findings provide clear evidence of the link between the secretory and endocytic pathways at the plasma membrane.

Clathrin Is Essential for Stomatal Function

Our findings connect clathrin function to stomatal behavior in a manner that is counterintuitive. Through the identification of the has1 mutant as a new chc1 mutant allele, we show that chc1 mutant plants have stomata that are impaired in their closure as well as reopening, indicating a requirement for clathrin-mediated transport in guard cell function (Fig. 4). While the chc1 stomata can open, their defective movement indicates a misregulation that has physiological effects on the plant, seen in the dehydration tolerance phenotype in both the chc1 and chc2 mutants: stomata of these mutants can close in response to dehydration stress signals, but they remain closed for longer periods of time when compared with the wild type. This defective stomatal movement leads to an increase in water retention under water-deficient conditions, which is most easily explained by the impaired ability of chc mutants to reopen after stomatal closure. Our further analysis of gas exchange in the chc1 mutants, which have lower transpiration levels compared with wild-type plants, again corresponds to a defect in stomatal aperture regulation (Fig. 4).

Figure 6. chc1 and chc2 mutants are sensitive to changes in light and humidity due to defective stomatal function. A, Representative plants grown under control light (70 μmol m⁻² s⁻¹) or high light (150 μmol m⁻² s⁻¹) and control RH (60%) or high RH (100%). The mutant rosettes are smaller in lower RH conditions, particularly when in high light. The growth phenotype is rescued under high humidity and control light conditions. B, Graphical representation of mean rosette diameter for each genotype under the different growth conditions. C, Steady-state transpiration rates (mmol water m⁻² s⁻¹) in the chc mutant plants are lower than that of Col-0 in control and high-light conditions. In both B and C, asterisks indicate significant differences from the wild type in each condition (Student’s t test, where P < 0.005). Values are means ± s; n = 4 plants in each experiment, which was conducted three times.
Physiological Consequences of Defective CHC Function

The dehydration-tolerant phenotype observed in each of the chc1 and chc2 mutant alleles (Fig. 5) is consistent with previous findings that the chc1 mutants close their stomata in response to low concentrations of exogenous ABA (Plessis et al., 2011). These observations indicate that the plants either are better able to sense water loss or are deficient in their ability to closely regulate transpiration compared with wild-type plants. The chc mutant plants are smaller under our growing conditions compared with the wild type and are substantially smaller still when grown under high light and lower humidity (Figs. 5 and 6). Much like what has been demonstrated in the sup121 mutant, this reduction in growth is most likely the result of reduced photosynthetic activity due to the disrupted gas exchange/water regulation in the mutants, consistent with the reduced transpiration rates we observed (Fig. 6C). The growth phenotypes under these different water-stress conditions correlate well with the stomatal phenotype we observed in the chc mutants and mirror those of the sup121 mutant (Eisenach et al., 2012). Again, the similarities between the growth phenotypes of the endocytic (chc) and exocytic (sup121) mutants suggest common underpinning mechanisms behind their physiological functions. Together with the inhibited secretion in the chc mutants, we now have cellular and genetic evidence for a coupling of the SNARE and clathrin machinery that are major players in vesicle transport at the plasma membrane. This coupling is vital for effective stomatal function and has major downstream effects on plant development.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds were surface sterilized with 20% sodium hypochlorite for 20 min at room temperature and then washed five to six times in sterile water. Seeds were then stratified in the dark for 48 h at 4°C before use. Arabidopsis (Arabidopsis thaliana) mutants chc1-1 (SALK_112213), chc1-2 (SALK_103252), chc1-3 (SALK_018351), chc2-1 (SALK_028826), and chc2-3 (SALK_151638) in the Col-0 ecotype background were compared with the untransformed wild type (Col-0) in all experiments. After backcrossing to the wild type and segregation of the aba3-1 mutation, the has1 mutant was backcrossed twice to the wild type before analysis of mutant phenotypes.

Map-Based Cloning and Sequencing

For mapping, the leaf temperature of F2 progeny from crosses between aba3-11 and has1 aba3-1 was analyzed by thermal imaging, and DNA was extracted in a 96-tube format. The mapping interval was then determined after genotyping F2 plants using simple sequence length polymorphism markers, as described by Plessis et al. (2011). Genomic sequencing was carried out on the has1 aba3-1 mutant after two backcrosses to the aba3-1 mutant. DNA was isolated as described by Sechet et al. (2015), and sequencing was performed by TGAC using an Illumina HiSeq 2000 sequencer.

Water Stress Experiments

Rapid dehydration assays were carried out using 3-week-old plants grown in the glasshouse (18°C–20°C, minimum 13-h photoperiod). Four rosettes per genotype were cut from the root system, placed abaxial face up on a support of absorbent paper and aluminum foil, and weighed before being placed in a laminar flow hood at ambient temperature. Plants were subsequently weighed at regular intervals to determine the rate of water loss (North et al., 2005). For phenotypic analysis of chc mutants under drought conditions, plants were grown on soil under 10 h of light/14 h of dark (150 μmol m–2 s–1) at 18°C to 22°C. For each genotype, eight plants were watered every other day with 20 mL of water for 2 weeks, after which watering for four plants from each genotype was stopped for 12 d. Watering was continued for the other four plants from each genotype (Plessis et al., 2011). Images were taken every day after watering was stopped for the drought cohort, and on day 12, rosettes were collected and the fresh weight was measured. The rosettes were dried for 3 d at 60°C, and the dry weight of each was recorded.

Light and Humidity Growth Conditions

Single plants were grown from seeds in 6-cm pots and watered with tap water regularly for 3 weeks under either 70 or 150 μmol m–2 s–1 light intensity in short-day conditions (8 h of light/16 h of dark). In each of these light conditions, plants were either covered with a plastic dome to give greater than 98% RH or left uncovered (60% RH). Plants were imaged 20 d after germination.
Stomatal Aperture Measurements

Epidermal peels were mounted in a custom flow chamber and preincubated for 2 h in DB (10 mM MES-NaOH, pH 6.15, and 60 mM KCl). Sets of individual stomata were imaged throughout with continuous superfusion using a Leica SMD SP8 confocal microscope (Leica Microsystems). To induce stomatal closure, peels were treated with HB (10 mM MES-NaOH, pH 6.15, 0.1 mM KCl, and 6 mM CaCl2) for 20 min. To induce reopening of stomata, DB was introduced into the chamber to replace the HB. Stomatal apertures were analyzed from three independent experiments (n = 22–30 stomata per genotype) using ImageJ version 1.48p (www.ImageJ.org). Chloroplast movement was used to confirm that living stomata were measured. Apertures were normalized on a cell-by-cell basis to measurements taken at the end of the preincubation period.

Genotypic Analysis

To isolate genomic DNA, 5- to 10-d-old seedlings grown in liquid 0.05× Murashige and Skoog (MS) medium were collected in 2-mL Eppendorf tubes, flash frozen on liquid nitrogen, and ground to a powder. A volume of 500 μL of extraction buffer (0.2 M Tris-HCl, pH 8, 0.4 M LiCl, 1% SDS, and 25 mM EDTA, pH 8) was added to each sample and mixed by vortexing. Samples were centrifuged at maximum speed for 10 min, and the supernatant was transferred into 12-well plates. Transient transformations were carried out with the pTecG-2in1 vector (Karnik et al., 2013) following the protocol of Grefen et al. (2015). The inoculation solutions were added to the appropriate wells containing 3-d-old seedlings, and roots were imaged 3 to 4 d after co-cultivation. The GFP and YFP fluorophores were excited using 440- and 514-nm laser lines, respectively. After chromatic separation, GFP fluorescence was collected over 475 to 520 nm and YFP fluorescence was collected over 535 to 600 nm. Fluorescence images were rendered as 3D projections, and quantification of fluorescence intensities from the roots was calculated after subtracting the mean background fluorescence collected from similarly rendered projections of untransformed seedlings grown in parallel in each experiment (Karnik et al., 2013).

Steady-State Whole-Plant Transpiration Measurements

Wild-type and mutant Arabidopsis plants were grown on soil for 3 weeks under 70 or 150 μmol m⁻² s⁻¹ light in short-day conditions at 60% RH and 22°C. Using an LI-6400XT portable photosynthesis system (LI-COR Biosciences), measurements were collected every 10 s for 3 min using the whole-plant chamber to maintain the light, humidity, and temperature conditions in which each plant had been grown. Five plants of each genotype were measured, and mean values for the transpiration rates ± se (mmol water m⁻² s⁻¹) were plotted for each light condition.

Statistics

All data are reported as means ± se of n independent experiments unless noted otherwise. ANOVA and Student’s t test were carried out using SigmaPlot version 11.0 (Systat Software).

Accession Numbers

Accession numbers are as follows. For CHC1 (At5g11130) and associated T-DNA mutants: chc1-1 (SALK_112213), chc1-2 (SALK_103252), and chc1-3 (SALK_018351); for CHC2 (At3g08530) and associated T-DNA mutants: chc2-1 (SALK_028926) and chc2-3 (SALK_151638); for SYP122 (At3g11820) and the mutant sup121-1 (sup1/psn1-1) is a point mutation mutant with a premature stop codon in the open reading frame (Leyman et al., 1999; Collins et al., 2003; Sokolowski et al., 2008); for SYP122 (At3g52400) and the associated T-DNA mutant sup122 (SALK_021525).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Genotyping chc1 and chc2 mutant alleles.

Supplemental Figure S2. Tamoxifen treatment alters growth and root cell organization only in the HUB1 lines.

Supplemental Figure S3. Dehydration tolerance in two dominant negative HUB1 clathrin mutants.

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LITERATURE CITED

Abeliovich H, Darsow T, Emr SD (1999) Cytoplasm to vacuole trafficking of aminopeptidase I requires a t-SNARE-Sec1p complex composed of Til2p and Vps4p. EMBO J 18:6005–6016


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