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Research Article

Modulating secretory pathway pH by proton channel co-expression can increase recombinant protein stability in plants

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Eukaryotic expression systems are used for the production of complex secreted proteins. However, recombinant proteins face considerable biochemical challenges along the secretory pathway, including proteolysis and pH variation between organelles. As the use of synthetic biology matures into solutions for protein production, various host-cell engineering approaches are being developed to ameliorate host-cell factors that can limit recombinant protein quality and yield. We report the potential of the influenza M2 ion channel as a novel tool to neutralize the pH in acidic subcellular compartments. Using transient expression in the plant host *Nicotiana benthamiana*, we show that ion channel expression can significantly raise pH in the Golgi apparatus and that this can have a strong stabilizing effect on a fusion protein separated by an acid-susceptible linker peptide. We exemplify the utility of this effect in recombinant protein production using influenza hemagglutinin subtypes differentially stable at low pH; the expression of hemagglutinins prone to conformational change in mildly acidic conditions is considerably enhanced by M2 co-expression. The co-expression of a heterologous ion channel to stabilize acid-labile proteins and peptides represents a novel approach to increasing the yield and quality of secreted recombinant proteins in plants and, possibly, in other eukaryotic expression hosts.

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Abbreviations: **α1ACT**, α_1-antichymotrypsin; **CPMV-*HT***, cowpea mosaic virus hypertranslatable; **dpi**, days post infiltration; **ER**, endoplasmic reticulum; **GFP**, green fluorescent protein; **HA**, hemagglutinin; **PDI**, protein disulfide isomerase; **sec**, secreted; ***Sl*CYS8**, eighth domain of tomato cystatin

1 Introduction

Plant-based expression systems represent highly efficient eukaryotic alternatives to produce various complex recombinant proteins [1]. Advantages over traditional prokaryotic expression systems include the ability to fold complex heteromeric proteins and to perform mammalian-like post-translational modifications [2]. Compared to other commonly used eukaryotic hosts such as mammalian and insect cells, plant-based protein expression can offer reduced capital cost, simple and rapid transformation as well as reduced risk of contamination with animal pathogens. However, many of the challenges to recombinant protein production in insect and mammalian cells also apply to plant tissues and cells. Biochemical constraints of the expression host, particularly

those associated with protein post-translational processes, determine the overall stability, accumulation and quality of recombinant products.

Diverse host cell engineering approaches that have recently emerged with the maturation of synthetic biology have proved to be effective in increasing recombinant protein expression levels. Overcoming biochemical limitations on recombinant protein yield and quality may be achieved by modulating post-transcriptional or post-translational processes acting directly on the heterologous protein [3, 4], or on host cell physiology [5, 6]. These rational modifications, making use of both stable co-expression and transient co-transfection, have shown the potential of synthetic biology to modulate the eukaryotic host cell for increased recombinant protein production [7]. Transient expression in intact leaves of whole plants is one of the most effective methods for recombinant protein production in eukaryotes [1]. It offers a simple platform, permitting the co-expression of many proteins for host cell engineering, for the production of heteromeric recombinant proteins, and for the production of heterologous and novel metabolites [8]. For example, increased recombinant protein yield can be attained through co-expression of companion protease inhibitors to reduce the impact of endogenous proteolysis [4, 9]. In addition, glycosylation can be controlled through the transient reorganization of glycosyltransferases, thereby determining the quality of secreted recombinant antibodies [10].

The secretory pathway presents a particularly complex milieu for the maturation and modification of recombinant proteins including heterogeneous redox environments and proton concentrations between subcellular compartments. Despite the fact that the pH gradient across compartments has a central role in secretory function [11, 12], pH variations can induce significant conformational changes in protein structures [13] and aggregation status [14]. Perturbations in the pH gradient can also compromise proper glycosylation of maturing proteins [15] or slow down molecular traffic along the secretory pathway [16]. Proteins destined for secretion are synthesized in the endoplasmic reticulum (ER) with near neutral pH and downstream compartments become progressively more acidic [17, 18].

Considering the potential difficulties imposed by heterogenous pH on protein maturation and stability, we hypothesized that regulating pH in the plant secretory pathway would enhance the stability and, potentially, the accumulation of certain recombinant polypeptides. The M2 protein from members of the Orthomyxoviridae forms a tetrameric pH-activated proton-selective ion channel [19] that plays multiple roles in the life cycle of orthomyxoviruses. One of these roles is to maintain a high pH in the trans-Golgi network of infected cells to prevent premature conformational change of hemagglutinin (HA) [20]. The M2 protein contains a 20-residue transmembrane domain helix [21] that is destabilized by low pH resulting in the opening and activation of the channel [19]. In the present study we have monitored the effect of M2 expression on the intracellular pH of plant leaf cells through the use of a pH-sensitive variant of the green fluorescent protein (GFP), ratiometric pHluorin [22]. Transiently expressing pHluorin targeted to specific organelles of the secretory pathway, we show the potential of M2 co-expression to improve the stability and accumulation of recombinant proteins, including influenza hemagglutinin, in the plant cell secretory pathway. These findings demonstrate the practical potential of ectopic pH regulation of the cell secretory pathway to positively impact the stability and accumulation of pH-susceptible recombinant proteins.

2 Materials and methods

2.1 Plasmid constructs

The coding sequences of ratiometric pHluorin (GenBank accession AF058694) and Influenza A/New Caledonia/20/1999 (H1N1) M2 (GenBank accession HQ008884.1) were placed within cowpea mosaic virus (CPMV)-*HT* regulatory sequences [23, 24] downstream of a double cauliflower mosaic virus (CaMV) 35S promoter and followed by the nopaline synthase (*nos*) terminator. To enable transit through the plant secretory pathway, gene constructs for secreted (sec)-pHluorin and ER-pHluorin contained a 5′ sequence encoding the protein disulfide isomerase (PDI) signal peptide [25]. Nucleotides encoding a carboxy-terminal SEKDEL motif were added to the 3′ end of the gene to retain the protein in the endoplasmic reticulum (ER). For targeting to the trans-Golgi we used the N-terminus of *Glycine max* α-1,2 mannosidase I consisting of the transmembrane domain, 29 residues of the cytoplasmic tail and 54 amino acids of the luminal stem region [26]. Seven codons were removed from the sequence encoding the transmembrane domain by PCR amplification to produce the Man99-pHluorin construct for localization to the cis-Golgi [26]. The influenzavirus A M2 gene (synthesized by DNA 2.0, USA) included a native signal peptide-coding sequence and a mutation changing the alanine (A)-30 to a proline (P) residue was generated by the Quickchange mutagenesis method (Agilent Technologies, Mississauga ON, Canada) to generate an inactive control [19]. Expression cassettes were transferred into the plant binary vector pCambia 2300 (CAMBIA, Canberra, Australia) containing an expression cassette for the co-expression of the silencing suppressor p19 [27]. A construct for the expression of a secreted tomato cystatin *Sl*CYS8–α_1-antichymotrypsin (α1ACT) fusion protein model, separated by a rigid linker, has been previously described [28].

The coding sequences for Influenza HA proteins (synthesized by DNA 2.0, USA) were also placed within CPMV-*HT* regulatory sequences downstream of a double

CaMV 35S promoter and followed by the *nos* terminator. HA sequences from B/Brisbane/60/2008 (GenBank accession FJ766840), from A/California/07/2009 (H1N1; GenBank accession FJ966974), and from A/Perth/16/2009 (H3N2; GenBank accession GQ293081) were preceded by DNA coding for the PDI signal peptide, while HA from A/Indonesia/05/2005 (H5N1; GenBank accession ABP51969) was cloned with its native signal peptide-coding sequence. Expression cassettes were transferred to a modified pCambia 2300 vector containing a cassette for the expression of the p19 silencing suppressor [27]. The expression cassette for influenza B HA was additionally inserted between bean yellow dwarf virus replication associated elements for DNA amplification [29].

2.2 Plant-based expression

pCambia vectors were maintained in *Agrobacterium tumefaciens* strain AGL1, which was transformed by heat shock. The bacterial cultures were grown to stable phase in Luria-Bertani medium supplemented with the appropriate antibiotics, and pelleted by gentle centrifugation. Following resuspension in infiltration medium (10 mM MES (2-[*N*-morpholino]ethanesulfonic acid), pH 5.6, 10 mM $MgCl_2$) to an OD_{600} of 0.5 and 2 to 4 h incubation at ambient temperature, suspensions were pressure infiltrated into *Nicotiana benthamiana* leaves using a needleless syringe. Co-agroinfiltration of pHluorin, fusion protein or HA constructs with M2 was at a 4:1 ratio with the target protein infiltrated at the same bacterial density in the presence or absence of the M2 construct. Leaf tissue was harvested five days post infiltration (dpi) except where indicated otherwise. To minimize the variability of protein expression levels from leaves of different age [4], the experiments were conducted to allow sampling from at least three different leaves from three plants per treatment.

2.3 Protein extraction

To extract soluble proteins from leaf material, infiltrated leaf tissue was harvested as leaf discs representing 160 mg of control-infiltrated tissue, and homogenized by disrupting tissue with ceramic beads in a Mini-Beadbeater apparatus (BioSpec, Bartlesville OK, USA). Fusion protein-expressing tissue was extracted in three volumes of phosphate-buffered saline (PBS), pH 7.3, containing 5 mM EDTA, 0.05% v/v Triton X-100, and complete protease inhibitor cocktail (Roche Diagnostics, Laval QC, Canada). Leaf lysates were clarified by centrifugation at 20 000 g for 20 min. HA-expressing tissue was extracted in three volumes of 50 mM Tris buffer, pH 8.0, containing 150 mM NaCl, 0.1% v/v Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), and lysates were clarified by centrifugation at 10 000 g for 10 min.

2.4 Western blotting and densitometric protein quantification

pHluorin and the SlCYS8–α1ACT fusion protein were detected by Western blotting onto nitrocellulose membranes following electrotransfer of leaf proteins resolved by SDS-PAGE under reducing conditions. Non-specific binding sites were blocked by incubation in blocking solution (5% w/v skim milk powder in PBS, containing 0.025% v/v Tween-20), which also served as antibody dilution buffer. pHluorin was detected with anti-GFP monoclonal antibodies (Clontech Laboratories, Mountain View CA, USA used at 1:5000 dilution), followed by goat anti-mouse alkaline phosphatase(AP)-conjugated secondary antibodies (Sigma-Aldrich, Oakville ON, Canada used at 1:5000 dilution). *Sl*CYS8 was detected with rabbit anti-*Sl*CYS8 polyclonal serum [4] at 1:5000 dilution followed by incubation with AP-conjugated secondary antibodies raised in goat (Sigma-Aldrich used at 1:5000 dilution). HA proteins were detected with strain-specific antibodies from the Therapeutic Goods Administration, Australia (B and H3 both at 1:20 000 dilution), the Centre for Biologics Evaluation and Research, Rockville MD, USA (H5 at 1:4000 dilution) and Sino Biological, Beijing, China (H1 at 1 µg/mL). Horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) were rabbit anti-sheep at 1:10 000 for B, H3 and H5 or goat anti-mouse at 1:7500 for H1. Colorimetric signals were developed with 5-bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium (Sigma-Aldrich). Chemiluminescent signals were obtained using the ECL Advance Western blotting detection kit (GE Healthcare, Baie d'Urfé QC, Canada). Densitometric analysis of the fusion protein signals was performed with the Phoretix 2D Expression software, v. 2005 (NonLinear USA, Durham NC, USA), on the Western blots digitalized with an Amersham Image Scanner (GE Healthcare). Densitometric analysis was performed on three independent biological replicates.

2.5 Imaging and data analysis

Transient expression of ratiometric pHluorin in fresh leaf tissue was imaged using a Nikon C1 confocal laser imaging microscope (Nikon, Melville NY, USA). Excitation scans were performed at 405 and 488 nm with the emission set at 515 nm using a 60× water-immersion lens. The power of each laser line and the gain were maintained at consistent levels between experiments so that the images were comparable. Image data were analyzed using the Open source software ImageJ (http://rsb.info.nih.gov/ij/) and background values, such as autofluorescence from chloroplasts, were subtracted from each image based on the average values of images acquired from uninfiltrated plants. Images or pixels with saturated intensities were eliminated or set to zero using a mask. Some pixels were

excluded by masking if their intensity values fell below a cut-off threshold and ratio values were generated through pixel-by-pixel calculations of intensities. Each treatment was evaluated with more than 15 pairs of images coming from three different plants and from leaves of the same morphological age.

2.6 Statistical analysis

Statistical analyses were performed using SAS, version 9.1 (SAS Institute, Cary NC, USA). Analysis of variance (ANOVA) using the General Linear Model (GLM) procedures was used to compare fluorescence ratios and yields of pHluorin expressed with or without M2. Contrast and LSD calculations were made when the ANOVA was significant at an α value threshold of 0.05. The relative abundance of intact fusions was compared using a mean comparison Student's *t*-test at $\alpha = 0.05$.

3 Results and discussion

3.1 pH changes along the cell secretory pathway

Ratiometric analysis of fluorescence is a precise way to measure small pH variations as ratios are independent of protein concentration, excitation intensity or cell artefacts [11, 30]. We used ratiometric pHluorin [22], a variant of GFP, as a tool to measure in situ pH variations in the secretory pathway of transiently transformed plant cells. To be able to measure pH variations along the pathway, we produced transient expression constructs to target pHluorin to the ER, to the cis-Golgi, to the trans-Golgi or to the apoplast (Fig. 1A). ER-targeted and apoplast-targeted pHluorin constructs were directed to the secretory pathway by the addition of a signal peptide of plant origin. Cis-Golgi and trans-Golgi pHluorin constructs were fused with the modified N-terminus of α-1,2 mannosidase

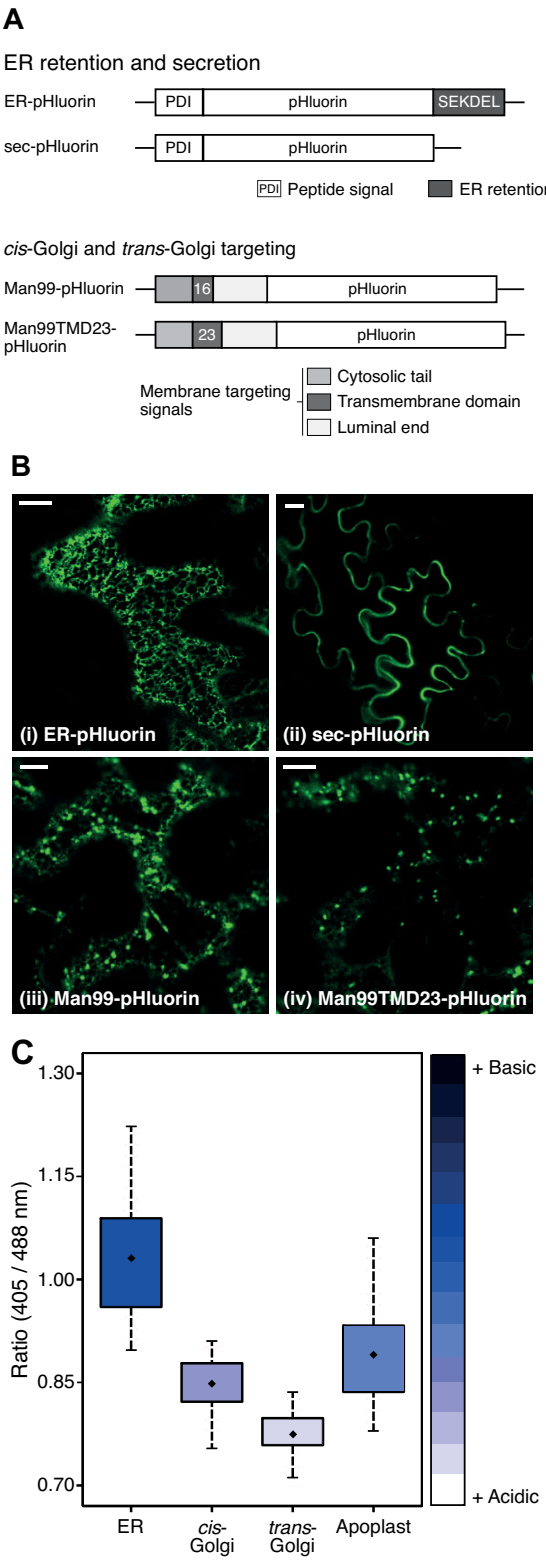

Figure 1. Measurement of plant cell secretory pathway pH by pHluorin targeting to different subcellular organelles. (**A**) Schematic representation of pHluorin constructs. ER-pHluorin contained an N-terminal signal peptide from protein disulfide isomerase (PDI) and a C-terminal (SEKDEL) ER-retention sequence. sec-pHluorin possessed just the PDI signal peptide for transit through to the apoplast. Man99-pHluorin had a version of the Man99 targeting signal for localization in the cis-Golgi with a 16-amino acid transmembrane domain. Man99TMD23-pHluorin had the full 23 amino acid transmembrane domain targeting the protein to the trans-Golgi. (**B**) Representative confocal microscopy images of transient expression of pHluorin in living *N. benthamiana* leaves. (i) Cell expressing the ER-retained pHluorin, showing a typical interconnected network pattern. (ii) Apoplastic accumulation of sec-pHluorin on the edges of the cell. (iii) Golgi stacks of the cis-Golgi highlighted by Man99-pHluorin. (iv) Late Golgi vesicles of the trans-Golgi identified by Man99TMD23-pHluorin. Bars = 10 µm. (**C**) Excitation ratios of pHluorin targeted to compartments of the plant cell secretory pathway. Ratios were calculated by comparing 15 pairs of images of epidermal leaf cells from three plants expressing pHluorin, excited at 405 and 488 nm. Small diamonds represent average ratios; boxes contain 95% of the data; bars represent the lowest and highest extreme values.

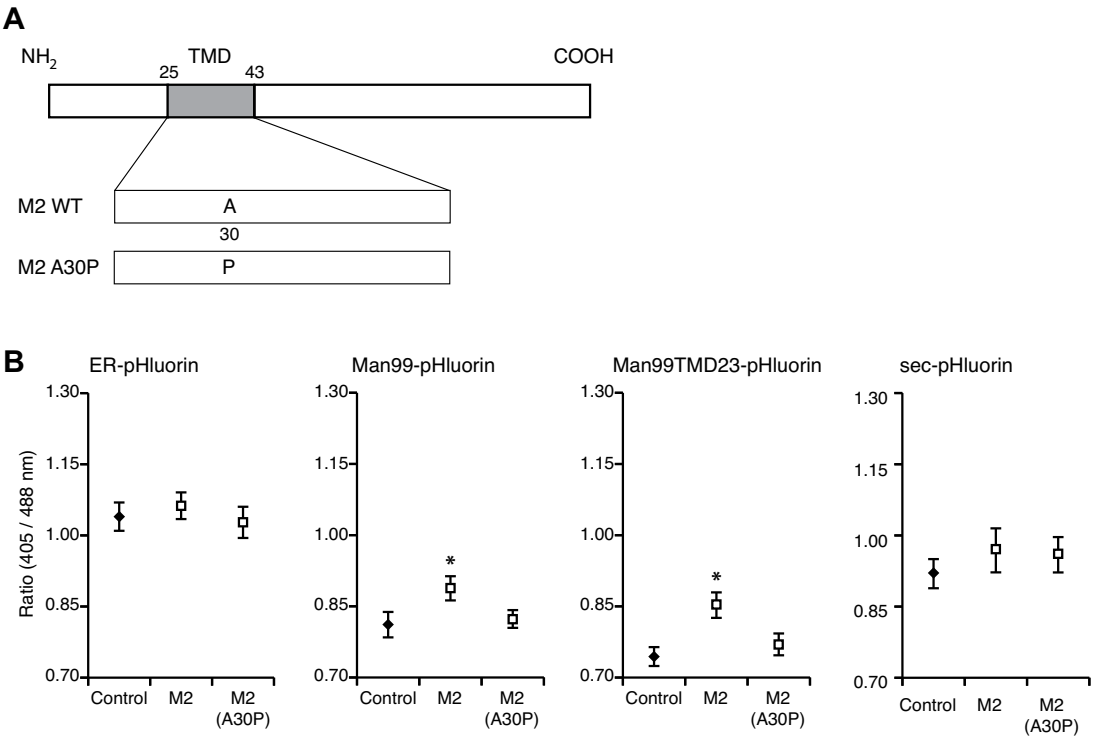

Figure 2. Impact of M2 ion channel co-expression on pH of secretory pathway compartments. (**A**) Schematic representation of the M2 constructs used in this study. The transmembrane domain (TMD) is expanded to show the position of the A30P mutation that renders the channel inactive. (**B**) Ratiometric fluorescence analysis of pHluorin targeted to the different cell compartments. Average ratios were calculated by comparing images from epidermal leaf cells expressing pHluorin, excited at 405 and 488 nm. Values are the mean of 15 replicates from three different plants ± SE. Asterisks indicate significantly different values ($P<0.05$).

I, a membrane-bound enzyme of the secretory pathway. Saint-Jore-Dupas and co-workers [26] showed by fluorescence microscopy that the Man99 membrane targeting signal was observed partially in the ER but mostly found in the cis-Golgi of *N. benthamiana* leaf cells. The same authors showed that the addition of seven amino acids in the transmembrane domain of Man99, yielding Man99TMD23, changed the localization of the protein to almost exclusively the trans-Golgi [26].

Confocal microscopy confirmed that transient agroinfiltrated pHluorin constructs were expressed and that pHluorin protein accumulated in different subcellular compartments. Fluorescence was sufficient at both excitation wavelengths for ratiometric analysis of *N. benthamiana* leaves (Fig. 1B). Epidermal cells expressing the ER marker protein (pHluorin-SEKDEL) showed a typical interconnected network pattern of the cortical ER [18], while secreted pHluorin accumulated on the edges of the cell in the apoplast. The Golgi-targeted proteins showed fluorescent patterns characteristic of the cis-Golgi and trans-Golgi patterns previously described in detail for several Golgi-localized GFP fusions in plants [26].

The excitation spectrum of pHluorin is bimodal and shows a reversible switch between the two excitation maxima at pH 7.5 and 5.5, resulting in ratiometric responsiveness within the pH range that characterizes the plant secretory pathway [18, 31]. While emission remains constant at 515 nm, excitation is strongest at 488 nm in acidic conditions whereas at pH closer to neutral the excitation maximum switches to 405 nm. Therefore, a low 405/488 nm ratio is indicative of acidic pH environment whereas a higher ratio indicates a more basic pH. As expected the ratio in the ER was the highest, indicating a more neutral pH, while the lowest observed ratio was in the trans-Golgi, revealing an acidic pH (Fig. 1C). Calibration of pHluorin in vitro has shown that the pH of the secretory pathway in agroinfiltrated *N. tabacum* leaves varies from the near neutral ER to a pH of approximately 6.0 in the trans-Golgi [18]. Apoplastic pH is known to be highly variable depending on environmental conditions [32] and we observed higher variability in this compartment of the cell compared to other compartments.

3.2 Impact of M2 on pH along the secretory pathway

To determine whether a transiently expressed proton channel can be used to modulate the pH of the secretory pathway of plants, we co-agroinfiltrated *N. benthamiana* leaves with Influenzavirus A M2 with the pHluorin con-

structs. An inactive M2 mutant was used to confirm that effects on pH were specific to proton channel activity (Fig. 2A). The A-30 to P (A30P) mutation eliminates ion channel activity without affecting the tertiary structure or appropriate transport to the plasma membrane [19].

M2 is co-translationally inserted into the ER membrane, transported along the secretory pathway to the plasma membrane [16], and is generally activated when reaching a cell compartment below pH 6.2 [20]. We reasoned that M2 would act to modulate the pH of the secretory pathway, wherever it is activated along the plant secretory pathway. Ratiometric fluorescence analysis 5 dpi confirmed that functional ion channel co-expression specifically modified the pH of the plant secretory pathway (Fig. 2B). No discernible pH change was observed in the ER in the presence of M2, which could be expected as the ER lumen is known to be close to neutral and, therefore, above the pH at which M2 is activated. On the other hand, while there was no impact of the M2 A30P mutant, a significant increase in the pH of both regions of the Golgi apparatus was seen with wild-type M2, with the highest impact observed in the trans-Golgi (Fig. 2B). Increased fluorescence ratios in the presence of M2 indicated that the ion channel was activated by the low pH of these organelles and that this activation resulted in a discernable change in pH. Although the apoplast is also slightly acidic, we were not able to see any difference between the presence and absence of M2, probably due to the high pH variability of this subcellular compartment [32]. Although we observed an effect suggestive of high proton channel activity in the Golgi, we did not observe any pH variation in the cytoplasm upon M2 co-expression (Supporting information, Fig. S1), which was not surprising given the strong pH buffering capacity of the cytoplasmic milieu [12].

A time course analysis of the effect of M2 on pHluorin excitation ratios was performed in subcellular compartments where there was an effect. Analysis of the timing of M2 expression showed the protein to be detectable after only 3 dpi and reach a maximum at 5 dpi, before finally decreasing (Supporting information, Fig. S2). As expected, no significant differences between ratios were observed for ER-targeted pHluorin in the presence of M2 at any time point. On the other hand, the impact of M2 in increasing pH of the cis-Golgi and trans-Golgi was detectable at 5 dpi and 4 dpi, respectively, and was maintained to at least six days despite an apparent decrease in M2 expression (Supporting information, Fig. S2). Taken together, these results show that it is possible to modulate the pH of the plant Golgi apparatus by transient co-expression of the M2 ion channel.

3.3 M2 co-expression stabilizes an acid-susceptible peptide linker

Fusion to a stabilizing protein domain is a strategy commonly used to increase the accumulation of recombinant proteins [33, 34]. An important consideration in the design of fusion constructs is the choice of the peptide linker sequence as this has substantial impact on both the performance [35–37] and stability [28, 37] of the fusion partners. The rigid linker $(EAAAK)_n$ can been used to separate proteins with control over the distance between fusion partners, potentially allowing for control over steric interference between the attached domains [38, 39]. This helical linker is frequently used in *Escherichia coli* [40] and mammalian cells [35], but is susceptible to cleavage in yeast [37] and plants [28]. It is also known to undergo auto-proteolytic cleavage at pH 6.0 to 7.0 in vitro [41]. The Golgi apparatus of plant cells is in this range of pH [18, 31] and in our *N. benthamiana* system we estimate a decreasing pH range of 6.5 to 6.0 along the cis-Golgi and trans-Golgi compartments. To investigate the possible impact of a general stabilizing effect on this linker by increasing the pH in the secretory pathway, we used a secreted fusion protein consisting of the eighth domain of tomato multicystatin, *Sl*CYS8 and a truncated version of human alpha-1-antichymotrypsin (α1ACT), separated by a $(EAAAK)_3$ peptide linker (Fig. 3A).

Increasing pH in the Golgi apparatus using M2 had a significant impact on the stability of the fusion protein containing the rigid linker. Western blotting confirmed that the fusion was not stable in the plant secretory pathway as the detection of free *Sl*CYS8 (10.7 kDa) showed that the two domains were separated (Fig. 3B). Co-expression of the fusion protein and M2 resulted in stabilization of the 55 kDa fusion product (Fig. 3B), resulting in a considerably enhanced accumulation of the intact fusion protein in the presence of the ion channel. Densitometric analysis of the Western blot signal showed that in the absence of M2 over 70% of the *Sl*CYS8 was detected in the free form, indicating that the majority of the fusion protein was cleaved. In contrast, M2 co-expression resulted in over 70% of the fusion product remaining intact (Fig. 3D). In terms of absolute accumulation of the intact fusion protein, a significant five-fold increase was observed in the presence of M2 (Fig. 3C). Together these results show that modulation of pH can have a positive impact on the accumulation of recombinant proteins and that peptide sequences can be stabilized through transient increases in pH along the secretory pathway.

3.4 M2 co-expression increases the yield of recombinant HA subtypes

Recombinant HAs are being developed as a subunit vaccines against influenza infection [42]. In plants, the expression of HA alone leads to the generation of influenza virus-like particles that bud from the plasma membrane [43] and such particles have been shown to elicit strong immune responses [44]. The expression of HA is complicated by the fact that different subtypes are differentially stable in acid conditions. A pH-dependent con-

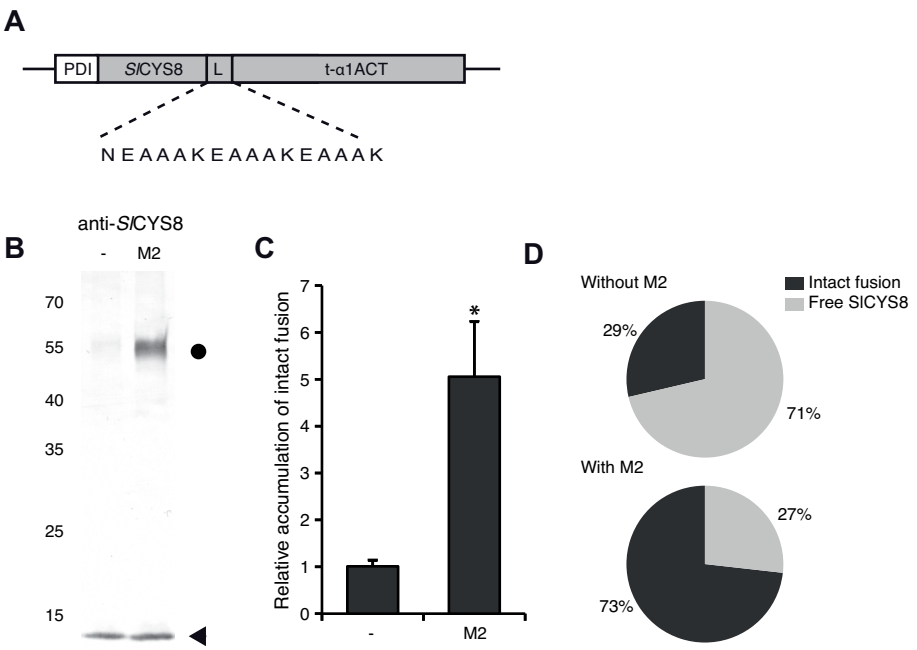

Figure 3. Effect of M2 ion channel co-expression on the stability of an acid-susceptible fusion protein. (**A**) Schematic diagram of the *Sl*CYS8-α1ACT fusion protein linked by an acid-susceptible rigid linker. PDI, signal peptide from protein disulfide isomerase; t-α1ACT, N-terminally truncated α1ACT; L, linker. (**B**) The fusion was co-expressed with or without M2. Equal volumes of soluble protein extracts to control for an impact of M2 expression on total protein content were loaded onto 12% w/v reducing SDS-PAGE followed by Western blotting for *Sl*CYS8 detection. The image presented is representative among three independent Western blots and the position of molecular size markers (kDa) is given on the left. The arrowhead points to the position of free *Sl*CYS8 and the closed circle to intact fusion. (**C**) Relative accumulation of the intact fusion co-expressed with M2 compared to the fusion expressed alone (given relative value of 1.0). Each bar is the mean of three independent values ± SD. The asterisk indicates a significant difference ($P<0.05$). (**D**) Percentage of intact fusion compared to free *Sl*CYS8, as inferred by densitometric analysis of three independent Western blots for the fusion expressed alone or along with M2.

formational change that activates membrane fusion during infection [45] is undesirable during transport to the plasma membrane and is prevented during HA expression and transit through the secretory pathway, in part by the activity of M2 [20]. In addition, mutations that impart higher activation pH for conformational conversion can increase virus replication and growth [46, 47]. We reasoned that M2 co-expression may result in increased HA yield by preventing premature conversion to the fusogenic state in the acidic Golgi, in a manner similar to the function of M2 during influenza infection.

Increasing pH in the plant cell secretory pathway by M2 ion channel co-expression had a marked effect on acid-labile HA subtypes (Fig. 4). Expression levels of HA from influenza B and H3 from influenza A were substantially increased by M2 co-expression. On the other hand, the yield of H1 and H5 were barely influenced by the presence of the ion channel. It has previously been shown that B and some H3 HA subtypes have higher activation pH values than most influenza A HA subtypes [48]. Therefore, during recombinant expression, conversion to the fusogenic form in the acidic Golgi could be limiting their accumulation at the plasma membrane and ectopically increasing pH in the Golgi above that of the activation pH prevents this conversion, increasing accumulation. Since M2 co-expression does not affect H1 and H5 levels, we presume that the lower activation pH for conformation conversion in these subtypes is not reached in the plant endomembrane system.

4 Concluding remarks

In this study, we have successfully modulated the pH of the plant secretory pathway through the expression of an orthomyxovirus M2 ion channel. To demonstrate this, we have used transiently expressed pHluorin as a unique tool to efficiently measure pH variations in the cell secretory pathway in situ. Co-expression of functional M2 results in a significant pH increase in the Golgi apparatus, as detected by pHluorin co-expression and a concomitant increase in the accumulation of recombinant proteins susceptible to low pH environments. Therefore, we have re-created in the plant cell the modification of cellular conditions employed by influenza viruses to improve the transit of hemagglutinin to the plasma membrane. To our knowledge, this is the first demonstration of the rational application of an ion channel to improve heterologous protein accumulation. While we work with plants as a host for recombinant protein expression, we expect this cell engi-

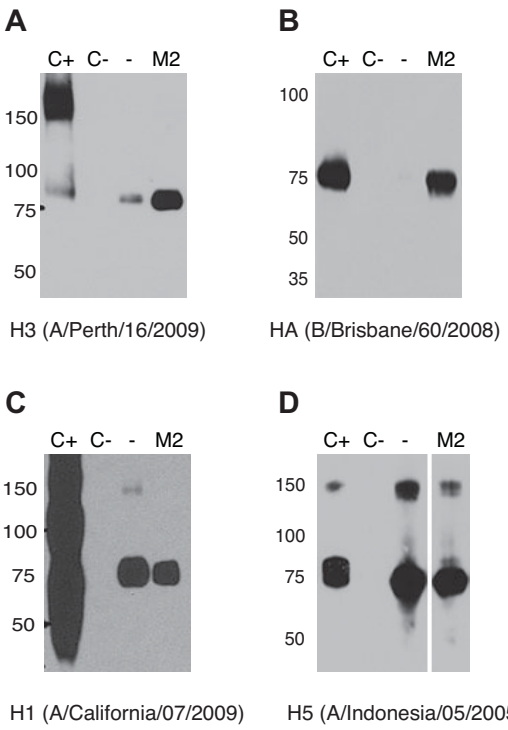

Figure 4. Effect of M2 ion channel co-expression on the expression of recombinant hemagglutinins. HAs were expressed with and without M2 co-expression and soluble proteins were separated by SDS-PAGE followed by Western blotting for each subtype. Positive loading controls (C+) consisted of semi-purified A/Wisconsin/15/2009 virus for H3 (**A**), semi-purified B/Brisbane/60/2008 virus for B HA (**B**), semi-purified A/California/7/2009 virus for H1 (**C**), and recombinant H5 from A/Indonesia/05/2005 (**D**). Negative controls (C–) were control protein extract from tissue infiltrated with empty expression vector and the images presented are representative Western blots of samples averaged across three biological replicates. The position of molecular size markers (kDa) is given on the left of each image.

neering approach to be applicable to eukaryotic expression systems in general.

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P. V. Jutras, M.-A. D'Aoust, M. M.-J. Couture, L.-P. Vézina, D. Michaud and F. Sainsbury are named inventors on a patent describing the use of ion channel co-expression to increase the yield of recombinant proteins. All other authors have no financial or commercial conflict of interest.

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