Intracellular traffic of proteins and endomembrane compartments in plant cells

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Two different types of P-ATPases co-operate to acidify the vacuole in plant cells.

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Two different types of P-ATPases co-operate to acidify the vacuole in plant cells.

The pH of the lumen of cellular organelles controls enzymatic reactions, protein sorting and vesicular traffic. In plant cells the pH in the lumen of endomembrane compartments is regulated by vacuolar V-ATPase and H⁺-pyrophosphatases (PPase). In epidermal petal cells of petunia a distinct P₃₅ATPase proton pump encoded by PH5 is required to acidify the central vacuole where anthocyanin pigments accumulate and to exhibit a reddish flower color. Here we report that PH5 works in concert with yet another P-ATPase (PH1) of the subfamily P₃B, which was only known in prokaryotes. Mutation of PH1 reduces vacuolar acidification in petals and results in a blue flower phenotype, similar to mutations in PH5 or the upstream transcription regulators PH3 and PH4. The combined ectopic expression of PH1 and PH5 is sufficient to restore vacuolar acidification in ph3 and ph4 petals and to acidify leaf vacuoles, indicating that these two proteins are the only two AN1/PH3/PH4 controlled factors required for vacuolar acidification and together constitute a universal vacuolar acidification machine. Split YFP experiments in plant cells and yeast two-hybrid assays indicate that PH1 and PH5 can physically interact and may operate as a heteromeric complex in vivo.

Flowers and fruits of most species are colored by the accumulation of anthocyanins pigments in the central vacuole of (sub) epidermal cells. The actual color depends on chemical modifications of the anthocyanins, the presence of co-pigments, and the pH of the vacuolar lumen (van Houwelingen et al., 1998). In petunia, mutations in a set of genes (PH1 to PH7) result in blue flower color as consequence of increased pH of the vacuolar lumen (de Vlaming et al., 1983; van Houwelingen et al., 1998). PH4 and PH3 encode transcription factors that, in concert with ANTHOCYANIN1 (AN1), AN2 and AN11, activate >10 subordinate genes, including PH5 which encodes a P₃₅-ATPase proton pump that resides in the vacuolar membrane (Verweij et al., 2008). Here we show that PH1 is another target of the AN1-PH3-PH4 complex and encodes a P₃B-ATPase that also resides in the tonoplast and interacts with PH5. PH1 and PH5 together are sufficient for (rescue of) vacuolar acidification in ph3, ph4 and an1 petals and in leaf cells where these genes are not normally expressed.
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To characterize PH1, we isolated a transposon-tagged allele, by crossing line R67 which harbors the stable \(ph1^{R67}\) allele, with a \(PH1\) line (W138) in which \(dTPH1\) elements transpose frequently. Among ~7000 \(PH1^{+/−}\) progeny with red colored flowers we identified one unstable \(ph1\) mutant bearing purplish flowers with occasional revertant (red) sectors (Fig. 1A). Selfings of this plant and backcrosses to a \(ph1\) line confirmed that it harbored a new (unstable) \(ph1\) allele and also yielded two independent revertants. Molecular analysis of the ~10 genes that are activated by AN1, PH3 and PH4, showed that the occurrence of the unstable \(ph1\) allele coincided with the insertion of a \(dTPH1\) transposon in the \(CAC7.5\) gene, 16bp downstream the start codon (Fig. 1B and 1D). In the two independent revertants (\(PH1^{R1}\) and \(PH1^{R2}\)) this \(dTPH1\) element excised and created a 6-bp footprint that restored the reading frame, whereas the insertion was maintained in mutant siblings. Furthermore, lines with stable \(ph1\) alleles (V23, V38, V48, R67) contain at the end of the \(CAC7.5\) coding sequence either a 7- or an 8-bp insertion that resembles a transposon footprint (Fig. 1D). Expression of the entire \(CAC7.5\) coding sequence from the constitutive \(35S\) promoter in a \(ph1\) mutant (V23xR170) rescued the (red) flower color and pH of petal homogenates (Fig. 1E). Taken together, these data prove that \(CAC7.5\) is encoded by the \(PH1\) locus.

\(PH1\) mRNA is expressed the limb and tube of the flower corolla and the ovary, with the highest expression in the growth stages of the bud (stage 3-4) and open flowers (stage 6), but not in other (partially) pigmented organs, such as anthers, and the pistil, nor in sepals, leaves, stem or roots (Fig. 1E). Furthermore, \(PH1\) is expressed in developing seeds, which accumulate condensed tannins, but not anthocyanins, about 15 days after pollination (dap). Analysis of mutants seeds and petals revealed that \(PH1\) mRNA expression is controlled by \(AN1\), \(AN11\), \(PH3\) and \(PH4\), as, but is independent from \(PH2\), or \(PH5\) and, hence, of vacuolar pH (Fig. 1H). Thus, the expression pattern and genetic regulation of \(PH1\) is highly similar, if not identical, to that of \(PH5\) (Verweij et al., 2008).
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**Figure 1. Isolation and characterization of PH1.** (A) Flowers the R67xW138 hybrid with a PH1+ phenotype (left) and mutant sibling harboring the mutable ph1L2164 allele. Note the large revertant sector with a red color. Anthocyanins in this background are cyanidins. (B) Schematic representation of the PH1 gene structure. Rectangles denote exons, the red triangle the dTPH1 insertion in ph1L2164 and the black bar the small insertions in the phl lines R67, V23, V42 and V48. (C) Seeds of the PH1+ (left) and ph1L2164 plants (right) shown in (a) (D) Sequence of the PH1+ allele compared to that of the insertion allele ph1L2164 and two independent revertant alleles (top), and the small insertions in the phl alleles in lines R67, V23, V42 and V46 (bottom). (E) Phenotype of phl flowers in the F2 hybrid (V23xV30) and of the transgenic expressing 35S:PH1 in the same background. Anthocyanins in this background are malvidin and petunidin resulting in a violet (PH1) of blue color (phl) (F) RT-PCR analysis the expression of PH1 during flower development and in different plant organs, and analysis of the genetic control of PH1 expression in flowers of different mutants. (G) Phylogenetic tree of different P-ATPase proteins representing the subfamilies 2A, 3A, and 3B. Prefixes indicate the species of origin: Ph, Petunia hybrida; Pt, Populus trichocarpa; Vv, Vitis vinifera; Re, Ricinus communis; Kp, Klebsiella pneumoniae; Ec, Escherichia coli; Csp, Citrobacter sp.; Pp, Physcomitrella patens; Dr, Danio rerio; At, Arabidopsis thaliana; Os, Oryza sativa

PH1 encodes a protein of 942 amino acids with high similarity to a bacterial magnesium transporters, such as MgtA of E. coli and Salmonella (Supplemental Fig S1). MgtA and MgtB of various bacteria constitute the P_3B-ATPase subfamily, which was thought to be specific for prokaryotes (Kuhlbrandt, 2004). Therefore PH1 represents the first identified eukaryotic member of this group. Interestingly, database searches revealed that the grape, peach, and poplar genomes contain PH1-homologs, but are missing in the genomes of Arabidopsis (Brassicaceae), rice and maize (monocots belonging to Poaceace) and more primitive plants, like Physcomitrella, (Bryophytes), Selaginella (Lycophytes) and Chlamydomonas (green algae) (see Fig 1g). This indicates that PH1 has an unusual evolutionary history and may have been gained through horizontal transfer early during angiosperm evolution, or has been very frequently lost in many angiosperm species/clades as well as in their non-flowering ancestors.

To determine where PH1 resides in petunia cells, we expressed PH1 proteins with a GFP-tag on either the N- or C-terminus from the 35S promoter in transgenic phl plants (35S:GFP-PH1 and 35S:PH1-GFP). Although both constructs rescued the wild type flower color and petal pH in transgenic ph1 plants they proved deceptive reporters, because in 35S:PH1-GFP tissue we could not detect the PH1-GFP protein with anti-GFP on immunoblots or GFP fluorescence, whereas in 35S:GFP-PH1 tissues anti-GFP detected only a cleaved protein of ~ 30kD that accumulated in a highly variable punctuate pattern in cells (Supplemental Fig. 2).
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Therefore we made a third fusion (35S:PH1-GFP-PH1) in which we inserted GFP in the second predicted cytoplasmic loop of PH1. When expressed in protoplasts from the petal epidermis, which faithfully reproduce the sorting of proteins in the intact tissue (Faraco et al., 2011), PH1-GFP-PH1 co-localizes with PH5-Cherry on the tonoplast that outlines the central vacuole with anthocyanins (tonoplast, Fig. 2A), while the plasma membrane marker RFP-AtSYP122 localizes on an adjacent membrane (plasma membrane, Fig. 2b). To test whether PH1-GFP-PH1 remains intact in plant cells, we transformed the 35S:PH1-GFP-PH1 construct into petunia ph1 mutants and we analyzed the fusion protein on immunoblots. (Fig. 2C). The PH1-GFP-PH1 protein is expressed at very low level (possibly due to instability of the fusion protein) but remains intact, confirming that it is a bona fide marker for the sub cellular localization of the PH1 protein. However, 35S:PH1-GFP-PH1 did not rescue the defects caused by ph1, which may result from the low expression level of the fusion protein or the disruption of the PH1 protein by the GFP insertion.

Constitutive expression of PH5 from a 35S:PH5 transgene is sufficient to rescue vacuolar acidification in ph5 mutants, but not in the regulatory ph3, ph4 and an1 mutants, indicating that besides PH5 additional proteins are required to acidify the vacuole (Verweij...
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et al., 2008). To assess whether co-expression of PH1 and PH5 is sufficient to acidify vacuoles, we generated transgenic 35S:PH1 plants in various genetic backgrounds. We observed that although 35S:PH1 efficiently complemented ph1 mutants (Fig. 1D), it could not complement ph3 mutants (Fig. 3A), even though the transgene was expressed at similar levels as the endogenous PH1 gene in wild type (Fig. 3B). Next we transformed 35S:PH1 in transgenic ph3 35S:PH5 plants and observed that transformants expressing both transgenes (ph3 35S:PH1 35S:PH5) had flowers with a wild type color and petal extract pH (Fig. 3A and 3B). Similar results were obtained in an1 mutants expressing both 35S:PH1 and 35S:PH5, although in this background only the pH of petal extracts was restored, but not anthocyanin synthesis (Fig. 3C), as this requires the induction of a different set of genes. The transformants in a ph4 background did not express the transgenes sufficiently high to restore the flower color, but nevertheless, we could detect partial restoration of the petal extract pH (Fig. 3D). Importantly, the pH of leaf homogenates of 35S:PH1 35S:PH5 complementants in the ph3 and an1 background was also lowered compared to untransformed controls and isogenic wild types (see Fig. 3A to 3C), but no obvious differences were seen regarding the shape or turgor of the leaves or trichome distribution. These observations indicate that PH1 and PH5 are the only target genes of the AN1-PH3-PH4 complex that are needed for acidification of vacuoles in epidermal petal cells and are sufficient to drive vacuolar acidification also in other cell types where they are normally not expressed.

To directly examine whether the alterations in the flower color and the petal extract pH are due to changes in the vacuolar pH in living cells, as assumed, we subjected petals of various fragments to in vivo 31P-NMR spectroscopy of vacuolar 31P (see methods). The results show that the observed alterations in flower color and the pH of homogenates do correlate with alterations of the vacuolar, and that pH values in crude homogenates are surprisingly close to those measured by NMR. Since both methods measure average vacuolar pH values for the entire corolla, they underestimate the pH shifts caused by these ph mutations, because these affect the epidermal cells only.
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Figure 3. Constitutive expression of PH1 and PH5 restores vacuolar acidification in ph3, ph4 and an1 mutants. (A) petal phenotypes of a PH3+ sibling, a ph3 mutant, and transgenic ph3 lines expressing 35S:PH1 or 35S:PH5. The green bars denote the pH of leaf extracts, violet and blue bars, the color and pH of petals and petal homogenates. (B) RT-PCR analysis of 35S:PH5 and 35S:PH1 transgen expression in the plants shown in (a). ACTIN mRNA was used as a constitutive control. (C) Flower of an an1 mutant expressing 35S:PH1 and 35S:PH5. The green bars denote the pH of leaf extracts, the white bars the pH of petal extracts. (D) Flower of a ph4 line expressing 35S:PH1 and 35S:PH5 with the same bluish color as a ph4 mutant. The green bars denote the pH of leaf extracts, the blue bars the color of the petals and the pH of homogenates. (E) In vivo vacuolar pH measure in leaves and petals by NMR spectroscopy based on vacuolar 31P

The high similarity of PH1 with bacterial Mg2+ transporters such as MgtA (Maguire, 2006), suggests that PH1 might export Mg2+ or another divalent cation from the vacuole to reduce the voltage gradient across the tonoplast and enable bulk proton transport by PH5. Similarly, in mammalian lysosomes it has been shown that a Cl-/H+ antiporter is necessary to facilitates acidification of the lumen (Graves et al., 2008) as in the absence of this, the bulk proton translocation cannot take place. Because it proved difficult to measure ion
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transport across the tonoplast of petal cells directly, we used genetic assays to examine whether PH1 can transport Mg\(^{2+}\). Although plasmid vectors with a PH1 cDNA proved toxic to E. coli, we could clone the PH1 open reading frame in low copy number plasmid behind a tightly regulated rhamnose-inducible promoter (Jong et al., 2010). However, induction of PH1 expression did not complement the MgtA mutation. Similarly, expression of the *E. coli* MgtA coding sequence in petunia *ph1* mutants did not rescue the *ph1* phenotype.

Since plasma membrane P\(_{3A}\)-ATPases were shown to form complexes consisting of 3 homodimers (Ottmann et al., 2007), we explored whether PH1 might acts in a (heteromeric) complex with PH5. Therefore, we expressed fusions of PH5 and PH1 proteins to the N- or C-terminal fragments of YFP from the 35S promoter in petal protoplasts. In these bimolecular fluorescence complementation (BiFC) assays (Walter et al., 2004), we observed fluorescence in protoplasts co-expressing PH1-YFP\(^{C}\) and PH5-YFP\(^{N}\). (Fig. 4A), while no fluorescence was ever observed in protoplasts expressing either one of the two constructs in combination with the complementary half of YFP (empty vector).

**Figure 4. Interaction between PH1 and PH5.** (A) Bimolecular fluorescence complementation in a transiently transformed protoplast from the petal epidermis expressing 35S:PH1-YFP\(^{C}\) and 35S:PH5-YFP\(^{N}\). Restored YFP fluorescence is shown in green, autofluorescence of anthocyanins in in blue. (B) Yeast two hybrid assays based on split ubiquitin. Fusions of the C-terminal (Cub) and N-terminal moiety (Nub) of ubiquitin were co-expressed in a yeast reporters and assayed for expression of the HIS3 and ADEReporter gene, visualised by growth in the absence of histidine and/ or adenin) and LacZ (visualized by blue staining in an X-gal overlay assay. growth on media lacking histidine. Nub-X nd pMETy-Cub denote the emptyy vectors used as negative controls.
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The restored YFP fluorescence signal was observed in small vacuole-like compartments that are distinct from the large central vacuole and highly similar to *vacuolinos*, which are a recently discovered cellular compartment via which vacuolar proteins, including PH5, PH5 and transiently resides before reaching the tonoplast of the central vacuole (Chapter 5).

To obtain independent evidence for the interaction between PH1 and PH5, we used a yeast two-hybrid system that is designed to assay interaction between membrane proteins and is based on the reconstitution of “split ubiquitin”. Preliminary results show that yeast cells expressing a fusion of PH5 and the C-terminal moiety of ubiquitin (Cub) together wit a fusion of PH1 of the N-terminal ubiquitin moiety (Nub) activated the HIS, ADE and LacZ reporter genes, indicating that PH1 and PH5 interact. These experiments revealed that PH5 can also interact with itself to form homodimers.

Here we identified a P$_{3B}$-ATPase protein, which were previously thought to be specific for prokaryotes, that acts in concert with the P$_{3A}$-ATPase proton pump to acidify the lumen of the vacuole where anthocyanins are stored. Our data show that these two constitute a novel system for the acidification of vacuoles, that presumably acts independent from the well-known vATPase and PPase pumps. That these two proteins can interact in vivo suggests that the actual pumping unit may be a heteromeric complex consisting of both P-ATPases. Given that plant plasma membrane P$_{3A}$-ATPases form complexes consisting of three homodimers (Ottmann et al., 2007), it is conceivable that PH5 is active in heterodimers together with PH1. This structure would be able to efficiently transport H$^+$ to the vacuolar lumen, possibly in combination with the extrusion of another cation (possibly Mg$^{2+}$) and avoiding in such way to increase the electrical gradient across the tonoplast.

The PH1/PH5 complex is a novel proton pumping system, because no P$_{3B}$-ATPases have so far been described in eukaryotes, and because no interaction with different pumps had been shown till now for P$_{3A}$-ATPases. As the interaction with PH1 is necessary for the activity of PH5, the diversification of PH5 from the rest of the P$_{3A}$ subfamily is not confined to the different localization of the protein in the cell (Verweij et al., 2008) but it has involved larger modifications in the protein which lead to a rather different mechanisms of activation.

Whereas V-ATPase and P-ATPase are widely conserved among plants and ubiquitously expressed, the PH1-PH5 system is both cell-type specific and species-specific, as several
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Plant species lack a PH1 homolog. PH5 and the Arabidopsis homolog AHA10 are required for the accumulation of proanthocyanidins (condensed tannins) in the seeds coat, presumably because they are needed to generate the electrochemical H⁺ gradient across the tonoplast that drives their vacuolar sequestration via the H⁺-antiporter TT12 (Debeaujon et al., 2001; Marinova et al., 2007). Even though PH1 is expressed simultaneously with PH5 during seed development, it is not essential for proanthocyanidin accumulation and PH5 activity, as ph1 seeds have a normal deep brown color, in contrast to ph5 seeds which are yellow, and because Arabidopsis lacks a PH1 homolog. Possibly the vacuoles in seed coats cells are less acidic than those in petals and a low activity of PH5 is sufficient to drive the H⁺ antiporter TT12. Anthocyanin transport, however, involves an ATP-driven MDR pump, at least in maize (Goodman et al., 2004). MDR proteins are powered by ATP rather than electrochemical trans-membrane gradient, explaining why ph mutations do not affect the accumulation of anthocyanins. Hence, the major role of PH1 and PH5 appears to be adjust the color of the petals, which is apparently essential in natural habitat, possibly to aid attraction of pollinators (Hoballah et al., 2007).

METHODS

Genetic stocks.
The petunia lines W225 (an1W225), W134 (an1W134), R144 (ph3V2068), R150 (ph4V22133), R160 (ph2iW2164), R159 (ph5R159) are derived by transposon insertions and excisions from the “wild type” line R27. The an2 mutant used was an F1 hybrid between line W240, harboring the unstable an2W240 allele. For protoplasts isolation we used the F1 hybrid M1xV30 (full wild type). These mutants as well as the ph1 mutant lines V23, V38, V48, R67 were present in the Amsterdam petunia collection.

As inbred lines are difficult to transform, for the production of transgenics we used hibrids between two lines carrying the same mutation. The ph3 mutant used for the generation of transgenics was the hybrid R143X R144, the ph4 mutant was the hybrid V64XR150 and the an1 mutant the hybrid W138XW242. The ph1 mutant used was the progeny of (V30xV23)xS. In this population, segregating for ph1 mutant and wild type plants, we could compare the effect of the introduction of PH1 constructs with the full wild type phenotype in isogenic background.
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All plants were grown under normal greenhouse conditions.

RNA analyses.
For all samples, total RNA was extracted from (organs of) two or three buds of a certain developmental stage from the same plant (in the case of transgenics) or different plants of the same line. RT-PCR analysis was carried out as previously described (Quattrocchio et al., 2006) using primers for PH5 (#1850 GTGGAATCCTTTGTCTTGGGTG and #1812 GAATCAATGTAAGTGAAGTCAGTCGG), PH1 (PH1-1801 GTGGAATCCTTTGTCTTGGGTG and #4001 CACCATGTGGTTATCAATATTTTCTG) and #4023 CAAGCATGATGCTGTAAGCAGC or GAPDH (#19 GTGGAATCCTTTGTCTTGGGTG and #20 CTGGAATCCTTTGTCTTGGGTG). For the analysis of the expression of the 35S:PH5 transgene we used primers #54 CACCATGTGGTTATCAATATTTTCTG and #4023 CAAGCATGATGCTGTAAGCAGC, and for the expression of the 35S:PH1 transgene primers #54 and #4023 CAAGCATGATGCTGTAAGCAGC.

Phylogenetic analysis.
The tree was constructed on basis of a ClustalW alignment of the full size sequences of the proteins by using a web-based version of the PHYLIP algorithm (http://www.phylogeny.fr/version2_cgi/simple_phylogeny.cgi). The P-ATPase sequences used were from the following species (Genbank accession numbers are given in brackets): Petunia hybrida (PH5: DQ334807.1); Populus trichocarpa (accession numbers, PH1-like: XM_002306475, P-ATPase H'; XM_002326589.1); Vitis vinifera (PH1-like: CBI41039 P-ATPase H': CBI35782); Ricinus communis (XM_002533519.1); Klebsiella pneumoniae (YP_002917472.1); Escherichia coli (YP_672334.1); Citrobacter sp. (ZP_04559666.1); Physcomitrella patens (XP_001760095.1); Danio rerio (NP_001001403); Arabidopsis thaliana (AHA1: CP002685.1, AHA2: CP002687.1, AHA4: Q9SU58, AHA10: NP_173169); Nicotiana plumbaginifolia (PMA9: AF156684.1, PMA2: AAA34052.1); Oryza sativa (AJ440220.1).
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**pH measurement of corolla crude extracts.**

Measure of the crude petal extract pH value was carried out as previously described (Verweij et al., 2008).

**Construction of transgenes.**

The entire **PH1** coding sequence including all 7 introns was amplified from genomic DNA using Phusion polymerase (Finnzymes) with primers #4001 CACCATGTGGTTATCCAATATTTCCTGTG complementary to start codon and #3917 complementary to the stop codon TAGGACTAAAGCCATGTCTTGAA. The fragment was cloned by TOPO reaction in pENTR/D-TOPO (Invitrogen) and from this clone was recombined into the expression vector pB7WG 2.0 (RU, Gent).

To produce the **PH1:GFP:PH1** fusion (in which the GFP sequence is inserted in the second cytoplasmic loop of the PH1 protein) we amplified 3 distinct fragments: 1) a **PH1** cDNA fragment spanning from aminoacid 1 to 194 of the protein (amplified from petal first strand cDNA from the line V30) with primers #5491 (GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGTGGTTACCCAATATT) and #5586 (GCATGGACGAGCTGTACAAGATTGTTCAAACTGAGGTACAGGTT), 2) the GFP coding sequence was amplified with primers #5584 (AGGTCAAAGATGTGACAGTGAAATGGTGAGCAAGGGGAGGA) and#5585 (AACCTGTACCTCAGTTGAACAATCTTTGTACAGCTGTCATGC) and 3) a genomic fragment (from V30 genomic DNA) of **PH1** (containing intron 4,5,6 and 7) with primers #5586 (GCATGGACGAGCTGTACAAGATTGTTCAAACTGAGGTACAGGTT) and #5492 (GGGGACCAGTTTGTTACAAAGACTGGGTAGGACTAAAGCCATGTCTTG). The three PCR products were then combined and re-amplified with the external primers #5491 and #5492 to yield one single PCR product.

This “tailored” fragment was recombined by BP reaction in pDONR P1-P2 (Invitrogen) and from this recombined in the expression vector pK2GW7 (RU, Gent).

The **PH1** fusion constructs for the Split-YFP experiments were constructed following a similar approach: for the the **YFP-N’** construct, we amplified the cDNA PHG1 fragment with primers #5019 (CAAGATCTATGTGGTTACCCAATATTTTTT) and #5583 (TCCTGCCCCCTTGCTCACCATTCTACCTGACACCATCTTTGAAACCT). The **YFP-N’**
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fragment with primers #5584 and #5702 (AACCTGTACCTCAGTTTGAACAAATGTCCTCGATGTTTGTGGCCGGAT), and the PH1 genomic fragment with #5703 (ATCCGCACAACATCGAGGACATTGTCAAACTGAGGTACAGGT) and #4633 (ATACGCAGCGCTAAAGCCATGTCTTGAAGACCAAATG). The combined amplification product was then digested with BglII and NotI and cloned in pENTR4 (Invitrogen) previously digested with BamHI/NotI. Subsequently, this was recombined into pK2WG7.

To build the YFP-C’ fusion construct, we amplified the cDNA fragment of PH1 with primers #5491 and #5704 (GTCGGCGAGCTGCACGCTGCCTCTACCTGACATCTTGAAACCT), the YFP-C’ fragment with #5705 (AGGTTCAAAGATGTGCAGGTAAGGCAGCGTGCAGCTCGACCAC) and #5585 (AACCTGTACCTCAGTTTGAACATCTTGTACAGCTCGTCCATGC) and the genomic PH1 fragment with primers #5586 (GCATGGACGAGCTGATCACAGTTTCAAACTGAGGTACAGGTT) and #5492.

**Protein analysis.**

Immunoblot analysis was done as described elsewhere (Verweij et al., 2008).

**Split ubiquitin assay.**

We used the mating based split ubiquitins system (mbSUS) (Obrdlík et al., 2004).

Due to the toxicity of PH1 and PH5 to E. coli, we used the in vivo cloning in yeast. To construct mbSUS fusions by recombination cloning in yeast, the open reading frame of PH1 has been amplified with primers #5746 (ACAAGTTTGTACAAAGAGCAGGCTCTCCAACCACCATGTGTTATCCAATATCTTGAAACCT) and #5747 (TCCGCCACACCAACCACCTTGTACAAAGAAGCGTG GTAAGCCATGTCTTTGAATACCAAATG) in order to obtain the ORF flanked by linkers B1 and B2. At the same time, we amplified PH1 with primers #5748 (ACAAGTTTGTACAAAGAGCAGGCTCTCCAACCACCATGTGCGAGGATCTTGAGAGAAG) and #5749 (TCCGCCACACCAACCACCTTGTACAAAGAAGCTGGGTAAACTGTATGCTGTTTTGT).
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In parallel, the vector pMetYCgate and the appropriate Nub vector (pXNgate21-3HA for PH5, pXNgate32-3HA for PH1) are cleaved with PstI/HindIII and EcoRI/SmaI, respectively.

The resulting B1-PH1-B2 (B1-PH5-B2) PCR products and the linearized vectors are used to co-transform either THY.AP4 or THY.AP5 yeast strains. Homologous recombination between B1 and B2 sequences of the PCR product and the linearized vector results in a circular vector harboring PH1 (or PH5) as a Cub-PLV or Nub fusion.

Transformants were selected on –Leu (CubPLV fusions) or on -Trp (Nub fusions) media. THY.AP5 strain is designed for the transformation with Nub fusions, the second strain THY.AP4 is designed for transformation with CubPLV fusions. Therefore we can test the interactions by mating approach in diploid cells.

**Protoplasts isolation, transformation and confocal microscopy.**

Isolation of protoplasts and transformation for transient expression of different GFP constructs was performed as described in (Faraco et al., 2011). GFP was imaged with a Zeiss LSM 710 or a Biorad 2000 confocal microscope using a 488 nm laser for excitation and mirrors set to select 505-530 nm emissions.

**Nuclear magnetic resonance spectroscopy.**

The $^{31}$P-NMR spectra were recorded on a standard broad-band 10-mm probe (AMX 600 spectrometer, Bruker Analytische Messtechnik, Rheinstetten-Forschheim, Germany) running with software TopSpin version 1.3. The recording was done at 242.9 MHz without lock, with a Waltz-based broad-band proton decoupling and a spectral window of 16 kHz. Chemical shifts were measured relative to the signal from a glass capillary containing 33 mM methylenediphosphonate (MDP), which is at 18.5 ppm relative to the signal from 85% H$_3$PO$_4$.

In-vivo $^{31}$P-NMR experiments were carried out by packing four flower limbs, or three leaves into a 10-mm-diameter NMR tube equipped with a perfusion system connected to a peristaltic pump in which medium [1 mM Mes-BTP (pH 6.1), 0.4 mM CaSO$_4$,] was aerated, thermoregulated (25 °C) and flowed at 10 ml min$^{-1}$. 
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Resonances assignment was obtained as described (Roberts et al., 1980; Kime et al.). Vacuolar pH was estimated from the chemical shift of inorganic phosphate (Pi) resonance. Standard titration curve relating d to pH were constructed on the d of 2.5 mM KH$_2$PO$_4$ dissolved in 25 mM KCl, 20 mM MgSO$_4$, 5 mM citrate acid, 5 mM malic acid buffered in the pH range from 5 to 6.5 with 10 mM MES-KOH. Ionic strength of the titration solution was similar to that of the cell sap of the limb flowers. The d values of Pi resonance measured in the cell extract of the different lines after the adjustment of the pH do not show significant differences.

The spectra used for the quantitative in-vivo analysis of vacuolar phosphate were determined using a 90° pulse angle and 6-s recycle time to give fully relaxed resonance. Its concentration in the vacuole was determined by comparing the resonance intensities with that of a glass capillary containing 33 mM MDP and previously calibrated against standard solutions (Spickett et al., 1992). The areas of the $^{31}$P vacuolar peaks were measured by Lorentzian line-shape analysis and the obtained values were referred to the percentage volume of the tissue in the NMR tube (Spickett et al., 1992).

**Measurement of Pi levels.**

Flower limbs were homogenized in 4 volumes of 10% (v/v) ice-cold trichloroacetic acid and centrifuged at 13000g for 15 min. Inorganic phosphate was determined in the supernatant using the Fiske and Subbarow method (Fiske and Subbarow, 1925).

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Figure S1. Alignment of the proteins encoded by PH1 and MgtA from E. coli. Sequence identity and similarity are indicated by black and grey shading respectively.
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Figure S2. Intracellular localization of GFP-PH1. (A) Localization of GFP-PH1 on small dots in wt petal protoplasts (B) Western blot analysis of wt petals agro infiltrated with 35S:GFP-PH1 and 35S:PH5-GFP.
REFERENCES


