The plasma membrane H⁺-ATPase gene family in Solanum tuberosum L. Role of PHA1 in tuberization

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Abstract

This study presents the characterization of the plasma membrane (PM) H⁺-ATPases in potato, focusing on their role in stolon and tuber development. Seven PM H⁺-ATPase genes were identified in the Solanum tuberosum genome, designated PHA1–PHA7. PHA genes show distinct expression patterns in different plant tissues and under different stress treatments. Application of PM H⁺-ATPase inhibitors arrests stolon growth, promotes tuber induction, and reduces tuber size, indicating that PM H⁺-ATPases are involved in tuberization, acting at different stages of the process. Transgenic potato plants overexpressing PHA1 were generated (PHA1-OE). At early developmental stages, PHA1-OE stolons elongate faster and show longer epidermal cells than wild-type stolons; this accelerated growth is accompanied by higher cell wall invertase activity, lower starch content, and higher expression of the sucrose–H⁺ symporter gene StSUT1. PHA1-OE stolons display an increased branching phenotype and develop larger tubers. PHA1-OE plants are taller and also present a highly branched phenotype. These results reveal a prominent role for PHA1 in plant growth and development. Regarding tuberization, PHA1 promotes stolon elongation at early stages, and tuber growth later on. PHA1 is involved in the sucrose–starch metabolism in stolons, possibly providing the driving force for sugar transporters to maintain the apoplastic sucrose transport during elongation.

Key words: Branching, PHA1, plant growth, PM H⁺-ATPase, potato, stolon elongation, tuber growth, tuberization.

Introduction

Plasma membrane (PM) H⁺-ATPases are integral membrane proteins that pump protons out of the cell, generating an electrochemical gradient of protons across the plasma-lemma. As a result, PM H⁺-ATPases provide the driving force for the transport of ions and metabolites through channels and transporters (Palmgren, 2001). This process is required for several physiological responses, such as cell expansion, phloem loading/unloading, stress adaptation, and plant growth and development (Michelet and Boutry, 1995; Palmgren, 2001; Gaxiola et al., 2007; Duby and Boutry, 2009; Schumacher and Krebs, 2010). PM H⁺-ATPases have been described in many plant species, such as Arabidopsis thaliana (Gaxiola et al., 2007), Nicotiana plumbaginifolia (Morsomme and Boutry, 2000), Oryza sativa (Baxter et al., 2003), Solanum
The plant PM H\(^+\)-ATPases are regulated at different levels. With respect to post-translational regulation, the mechanism involves the autoinhibitory action of the C-terminal domain that can be displaced by phosphorylation of the penultimate residue, a threonine, and the subsequent binding of 14-3-3 proteins, resulting in pump activation (Palmgren et al., 1991; Svennelid et al., 1999; Maudoux et al., 2000). More recently, it has been described that phosphorylation of other residues can also affect PM H\(^+\)-ATPase activity (Duby et al., 2009; Piette et al., 2011), suggesting that these proton pumps are controlled by a complex regulatory mechanism. Other factors can regulate PM H\(^+\)-ATPase activity. A novel interaction partner of PM H\(^+\)-ATPase named PPI (proton pump interactor) was identified in *A. thaliana* (PPI1; Morandini et al., 2002) and *Solanum tuberosum* (StPPI1; Muñiz Garcia et al., 2011); this protein increases the activity of the proton pump in vitro once the C-terminus has been displaced.

Several studies have indicated that regulation of PM H\(^+\)-ATPase also occurs at the genetic level. PM H\(^+\)-ATPases are encoded by five distinct gene subfamilies. The members of subfamilies I and II show ubiquitous expression profiles, while the expression of genes belonging to subfamilies III, IV, and V is restricted to specific tissues (Arango et al., 2003; Gaxiola et al., 2007). PM H\(^+\)-ATPase transcript levels are differentially regulated by environmental and hormonal signals, such as salt stress (Kalampanayil and Wimmers, 2001; Janicka-Russak and Klobus, 2007; Sahu and Shaw, 2009), dehydration (Surowy and Boyer, 1991), low temperature (Ahn et al., 1999), and exogenous application of hormones (Frias et al., 1996; Janicka-Russak and Klobus, 2007).

In potato (*Solanum tuberosum* cv. Désirée), two genes encoding PM H\(^+\)-ATPases (*PHA1* and *PHA2*) were isolated long ago by Harms et al. (1994). However, their physiological functions have not yet been elucidated. The economic importance of potato plants resides in the capacity to produce tubers. Potato tubers develop from stolons, which are underground stems. All aspects of tuber development are coordinated by a complex interaction of phytohormones and environmental signals, such as salt stress (Kalampanayil and Wimmers, 2001; Janicka-Russak and Klobus, 2007; Sahu and Shaw, 2009), dehydration (Surowy and Boyer, 1991), low temperature (Ahn et al., 1999), and exogenous application of hormones (Frias et al., 1996; Janicka-Russak and Klobus, 2007).

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At early stages of tuber formation, stolons display a cessation of elongation and the initiation of subapical radial growth, which is followed by a deposition of starch and storage proteins (Visser et al., 1994). During the transition from stolon to tuber, a switch from apoplastic to symplastic sucrose phloem unloading occurs (Viola et al., 2001). Phloem loading of sucrose in source leaves requires the activity of sucrose–H\(^+\) symporters, and apoplastic sucrose unloading in sink tissues involves sucrose–H\(^+\) and hexose–H\(^+\) symporters (Lemoine et al., 2013). These sugar transporters depend on the proton motive force generated by PM H\(^+\)-ATPases; therefore, these proton pumps might play an important role in stolon and tuber development.

This study presents a comprehensive overview of the PM H\(^+\)-ATPase gene family (PHA) in potato, including the identification of PHA genes and expression profiles to explore which PHA genes are possibly involved in tuberization. Based on the results of expression analysis, *PHA1* was chosen for further characterization by the development of transgenic plants.

### Materials and methods

#### Plant material

Soil-grown potato plants (*Solanum tuberosum* cv. Spunta) were cultivated in a growth chamber at 19°C or 22°C, under a 12 h or 16 h light photoperiod (4000 lux light intensity), or in a greenhouse maintained between 22°C and 24°C, under a 16 h light photoperiod. In vitro plants were obtained by micropropagation of virus-free single-node cuttings in Murashige and Skoog medium (MS medium; Proclon product M5190, PhytoTechnology Laboratories, Shawnee Mission, KS, USA) containing 20 g l\(^{-1}\) sucrose solidified with 0.7% (w/v) agar, under a 16 h light photoperiod (4000 lux light intensity) at 22°C.

#### Abiotic stress treatments

The first two fully expanded leaves detached from plants grown in soil, in a greenhouse, for 4 weeks were used for salt stress and drought treatments. Prior to stress treatment, leaves were placed in individual containers with water in a growth chamber at 22°C, under a 16-h light photoperiod, for 48 h, to allow the wound response components induced by excision to be restored to basal levels. Only the cut end of the petioles was immersed in the solution. For salt stress, leaves were treated with 250 mM NaCl. For drought treatment, water was removed from the containers and leaves were kept in air. For cold treatment, in vitro grown potato plants were exposed to 4°C, while control plants remained at 22°C, under a 16-h light photoperiod.

#### Stolon growth conditions and in vitro tuberization

Tuberization can be studied in vitro, reproducing the process occurring in vivo with the advantages of generating tubers in a controlled environment (Xu et al., 1998; Roumeliotis et al., 2012; Muñiz García et al., 2016). Single-node cuttings obtained from potato plants do not induce tubers when cultured in darkness on standard propagation media (MS medium plus 2% sucrose); however, increasing sucrose concentration (8% is the optimal concentration) increases the frequency of tuberization.

Shoot apices derived from in vitro plants were cultured on solid MS media containing 20 g l\(^{-1}\) sucrose for 2 weeks, in a growth chamber at 22°C, under a 16-h light photoperiod, prior to harvesting single-node explants. Nodal segments were grown on MS medium containing 20 g l\(^{-1}\) or 80 g l\(^{-1}\) sucrose (non-inducing and tuber-inducing conditions, respectively) solidified with 0.7% (w/v) agar, in a growth chamber in darkness at 19°C. In some experiments, the media was supplemented with hormones (5 μM GA\(_3\); 25 μM 3-indoleacetic acid, or 5 μM ABA; purchased from Sigma, St Louis, MO, USA) or the inhibitors of H\(^+\)-ATPase (1 mM sodium orthovanadate or 50 μM erythrosine B; purchased from Sigma). The single-node cuttings formed etiolated shoots/stolons that, under tuber-inducing conditions, developed tubers. For tuber-inducing conditions, the process was studied for 9–10 weeks. After this time of culture, most of the tubers were fully developed, and the rate of tuber growth decreased significantly.

All the experiments were performed at least three times independently, using 10–20 stolons per condition. A tuberizing stolon was defined as a stolon presenting visible subapical swelling or tubers.
Stolons presenting more than one subapical swelling/tuber were considered as one tuberizing stolon. A branched stolon was defined as a stolon presenting at least one branch 25 mm. The total stolon length was determined as the sum of the primary and secondary stolon lengths. Tuber weight was calculated as the sum of the weight of all the tubers divided by number of tubers obtained. If the stolon presented more than one tuber, only the fully developed tubers (minor diameter ≥4 mm) were used for weight determination, since, in most cases, the additional tubers were very small or undeveloped.

Tuberization in soil-grown plants

Yield determination was carried out on plants transferred to soil ex vitro and cultivated in a growth chamber at 19 °C, under a 12 h light photoperiod, for 10 weeks, in 0.5 liter pots with commercial soil mixture, in greenhouse, determination was carried out on plants obtained from seed tubers, grown in 0.8 liter pots with commercial soil mixture, in greenhouse, for 4 weeks.

Semi-quantitative reverse transcription–PCR (RT–PCR)

Semi-quantitative RT–PCR to determine PHA gene expression was performed as described in País et al. (2010) using the primers and reaction conditions shown in Supplementary Table S1 at JXB online. RT–PCR bands were quantified relative to the elongation factor 1-α gene (EF1-α) using ImageJ software (http://rsb.info.nih.gov/ij/).

Real-time quantitative RT–PCR (RT–qPCR) analysis

Relative expression of PHA1-3 and StSUT1 was determined by RT–qPCR. RNA was isolated and cDNA synthesis was performed as described in País et al. (2010). The cDNA was used as template for PCR amplification. The potato EF1-α gene was used as reference gene using the primers forward, ATGGGAAACGGATATGCTCCA; and reverse, TCTTATCCGAGCCGCTTCA. The primer sequences for PHA1–PHA3 were PHA1 5’ UTR forward, GGAAGAGAGGGAATGAGAAAGT; and PHA1 5’ UTR reverse, CTCCTTACGTGCTGTTGACC (PCR efficiency: 2.04); PHA2 forward, AGAAAAAGAGAGACACACGG; and PHA2 reverse, GACAAATCCCTTTCAATGGG; and PHA2 reverse, GTTGGTGTTGTGATGAGCG; and PHA2 reverse, CTCCTCTAGTTGTTGATCC (PCR efficiency: 1.97); PHA3 3’ UTR forward, GGTGGTGTGTGATGTACCACG; and PHA3 3’ UTR reverse, GAAAGGCTCCAGGAAACAGC (PCR efficiency: 2.02). The primer sequences for StSUT1 were obtained from Krügel et al. (2012) (LC-SUT1 fw, TTCCATAGCTGCTGGTGTTC; and LC-SUT1 rev, TACCAGAAATGGTCCTTCA). Reactions were performed in a final volume of 20 µl containing 4 µl of 5XHOT FirePol EvaGreen qPCR Mix Plus (Solis-BioDyne, Tartu, Estonia). The amount of cDNA used in each reaction was derived from 1 ng of total RNA for EF1-α, 10 ng for PHA1–PHA3, and 5 ng for StSUT1 (each cDNA sample was diluted accordingly). Reactions for EF1-α amplification were carried out under the following conditions: 50 °C/2 min (1 cycle); 95 °C/15 min (1 cycle); 95 °C/15 s; 60 °C/1 min; 72 °C/30 s (35–40 cycles). For PHA1–PHA3, reactions were carried out as follows: 95 °C/15 min (1 cycle); 94 °C/2 min (1 cycle); 58 °C/1 min (1 cycle); 72 °C/1 min (1 cycle); 94 °C/30 s, 58 °C/30 s; 72 °C/45 s (35–40 cycles). For StSUT1, reactions were performed with the following program: 95 °C/15 min (1 cycle); 95 °C/30 s, 61 °C/30 s, 72 °C/30 s (35–45 cycles). Amplification of a single product of the correct size for each gene was confirmed by agarose gel electrophoresis. The relative expression level was calculated using the 2ΔΔCt cycle threshold method (Livak and Schmittgen, 2001).

Cloning of the PHA1 gene from Solanum tuberosum cv. Spunta

The PHA1 coding sequence was obtained by RT–PCR from RNA isolated from potato (S. tuberosum cv. Spunta) flower buds using the primers PHA1 5’UTR Fw, GGAAGAGAGGGAATGAGAAAGT; and PHA1 3’UTR Rv, GCCGATAATGAAATGTATTAG. The amplified product was cloned into the pCR-Blunt II-TOPO vector (Thermo Fisher Scientific, Waltham, MA, USA), for sequencing.

Yeast complementation assay

The complementation assay was carried out using the Saccharomyces cerevisiae strain YAK2 (MATα, ade 2-101, leu2Δ, his3Δ200, ura3Δ, trplΔ63, lys2-801 pmal1Δ::HIS3, pma2Δ::TRP1) lacking the genomic copies of PMA1 and PMA2, the two endogenous H+-ATPase genes. Survival is possible by the expression of the PMA1 gene under the GAL1 promoter on a UR43-bearing centromeric plasmid (de Kerchove d’Exaerde et al., 1995). The YEplac181 plasmid (bearing the 2µ origin of replication and the LEU2 marker) containing the promoter region of the yeast PMA1 gene was used to express the different plant PM H+-ATPase genes. Colony PCR was performed to detect the presence of the corresponding plasmids in the YAK2 transformants. The transformed YAK2 strains were grown at 30 °C in a synthetic medium containing 0.7% yeast nitrogen base without amino acids, supplemented with all of the amino acids except those used for selection (His, Leu, Ura and Trp), and 2% glucose (MGl-His, Leu, Ura, Trp) or 2% galactose/1% raffinose (MGal-His, Leu, Ura, Trp). Solid media contained 2% agar. The media were supplemented with 20 mM K2HPO4 and buffered to a pH of 6.5. In addition, the YAK2 transformants were grown on MGl-His, Leu, Trp medium at pH 6.5, containing 0.1% 5-fluoroorotic acid (5-FOA) to counter-select the plasmid bearing the yeast PM H+-ATPase gene.

Generation of PHA1-OE transgenic potato plants

The PHA1 sequence was subcloned into the pBl121 binary vector downstream of the Cauliflower mosaic virus 35S promoter (35S CaMV). Transformation of potato discs was performed using Agrobacterium tumefaciens strain LBA4404 as described in Muñiz García et al. (2014). Regenerated plants carrying no plasmid but obtained from the same explants and by the same regeneration method were used as controls (wild type). All plants were obtained from the same tuber; therefore, regeneration controls and transgenic lines have the same genetic background. Three independent transgenic lines (L1, L2, and L3) were selected and characterized in this study.

PCR analysis was performed to confirm the presence of the transgene using genomic DNA as template and the primers PHA1 5’ UTR forward (GGAAGAGAGGAATGAGAAAGT) and PHA1 3’ UTR reverse (TTCCATAGCTGCTGGTGTTC; and LC-SUT1 fw, TTCCATAGCTGCTGGTGTTC; and LC-SUT1 rev, TACCAGAAATGGTCCTTCA). The transformed YAK2 strains were grown at 30 °C in a synthetic medium containing 0.7% yeast nitrogen base without amino acids, supplemented with all of the amino acids except those used for selection (His, Leu, Ura and Trp), and 2% glucose (MGl-His, Leu, Ura, Trp) or 2% galactose/1% raffinose (MGal-His, Leu, Ura, Trp). Solid media contained 2% agar. The media were supplemented with 20 mM K2HPO4 and buffered to a pH of 6.5. In addition, the YAK2 transformants were grown on MGl-His, Leu, Trp medium at pH 6.5, containing 0.1% 5-fluoroorotic acid (5-FOA) to counter-select the plasmid bearing the yeast PM H+-ATPase gene.

PM H+-ATPase activity

PM H+-ATPase activity was determined in purified membranes isolated by a two-step aqueous two-phase partitioning system as described in Olivari et al. (2000) from leaves of potato plants grown in soil for 60 d or stolons cultured in vitro on MS medium plus 8% sucrose for 3 weeks. The PM H+-ATPase activity was determined in 5 mM ATP, 5 mM MgCl2, and 10 mM PIPES pH 7.3, with the addition of 5 mM NaN3, 0.1 mM sodium molybdate, and 100 mM KNO3, in the absence or presence of 100 µM Na2VO4. The assay was performed in the presence of 0.01 mg ml−1 lysophosphatidylcholine (Papini and De Michielis, 1997). The assay was carried out using the plasma membrane samples (2.5 µg of protein) in a final reaction volume of 150 µl for 30 min at 30 °C. Released Pi was measured.
using the malachite green method (Hohenwallner and Wimmer, 1973). The PM H^+-ATPase activity was determined as the difference between the activity measured in the absence and presence of vanadate.

**Observation of stolon epidermal cell imprints**

A thick layer of clear nail polish was brushed onto the epidermis of each stolon. Once dried, the nail polish was peeled off, placed on a clear glass, and observed using a light microscope (Olympus BX41, Olympus Optical Co. Ltd, Tokyo, Japan). Photomicrographs of the imprints were obtained at ×200 magnification in the microscope.

**Starch content**

The samples (50 mg) were ground to a fine powder in liquid nitrogen and extracted three times with 10 ml of 80% ethanol, by boiling the samples in a 90 °C water bath for 20 min to dissolve sugars, dextrins, and tannins, and centrifuged at 1500 g for 5 min. The residues were washed three times with extraction buffer at a 1:5 (w/v) ratio with the same buffer. The activities were assayed in Whatman No. 40 filter paper. The filtrates were adjusted to pH 7.0 with NaOH and analyzed for glucose by the Somogyi–Nelson method (Nelson, 1994). Starch content was estimated by multiplying the glucose content by the glucose equivalent of 0.9. Results were expressed as g starch 100 g–1 FW.

**Cell wall invertase activity**

The pellet mix procedure was used to assess the activities of cell wall invertase (Albertson et al., 2001). About 50 mg of stolon tissue was homogenized in ice-cold extraction buffer at a 1:5 (w/v) ratio containing 50 mM Hepes (pH 7.5), 10 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg ml−1 trypsin inhibitor, 5 mM aminocaproic acid, 5 mM DTT, 0.1% (v/v) Triton X-100, and 2% (w/v) polyvinylpyrrolidone (PVP). The extract was centrifuged at 12 000 g for 10 min at 4 °C; the pellet was washed three times with extraction buffer without PVP and used for activity assay after a final resuspension in a final volume of 1 ml containing 125 mM Na-acetate buffer (pH 4.5), 100 mM sucrose, and 80 μl of the pellet mix. The reactions were incubated at 37 °C for 30 min. Reactions were stopped by boiling water for 1 min, centrifuged at 12 000 g for 5 min, and glucose was quantified in the supernatant according to the Nelson–Somogyi method (Nelson, 1994).

**Expression profile of PHA genes**

The expression profile of PHA genes in different organs was determined (Fig. 2A; Supplementary Fig. S2). PHA1, PHA2, and PHA3 were expressed in all organs, while expression of PHA4 and PHA5 was restricted to flower buds and flowers. PHA6 was expressed predominantly in flower buds and roots. PHA7 transcripts were not detected in any of the organs analyzed (not shown).

The mRNA levels of PHA genes were determined in stolons cultured in vitro. PHA1, PHA2, and PHA3 were expressed in stolons cultured under tuber-inducing conditions, while PHA4, PHA5, PHA6, and PHA7 transcripts were not detected (Supplementary Fig. S3). PHA1 and PHA2 were expressed at higher levels in early stages of tuber organogenesis than in the later stages, while PHA3 displayed an opposite expression pattern (Fig. 2B). The sucrose–H^+ symporter SfSUT1 showed an expression pattern similar to that of PHA1 and PHA2 (Fig. 2B). GA increased PHA1 and PHA2 expression in stolons, while no significant changes were observed for PHA3 (Fig. 2C); no significant changes in the expression of PHA genes were detected in response to auxin or ABA (Fig. 2C). PHA4, PHA5, PHA6, and PHA7 transcripts were not detected at any stage of tuberization, with or without hormone treatment (not shown).

**Results**

**Identification of PHA genes in potato**

A search in the Potato Genome Sequencing Consortium database, (http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml) revealed the existence of seven sequences presenting a high degree of homology with PM H^+-ATPases in the S. tuberosum Phureja genome, designated PHA1–PHA7 (Supplementary Fig. S1; Supplementary Table S2). Analysis of the protein primary structure revealed that the potato PM H^+-ATPases present the characteristic features of PM proton pumps: the C-terminal autoinhibitory domain, 10 transmembrane segments, the small cytosolic loop, between the second and third transmembrane segments, and the large cytosolic loop, between the fourth and fifth transmembrane segments (Supplementary Fig. S1). PHA6 is truncated at its C-terminus and presents a unique region of 88 amino acids, absent in the other members of the PHA family. PHA3 also presents a 34 amino acid sequence which is missing in the other PHAs. Scanning the PM H^+-ATPase protein sequences for motif analysis confirmed the presence of typical motifs for plant P-ATPases: TGES, crucial for ATPase activity, DKTGTLT, in which the aspartate is reversibly phosphorylated during catalysis, KGA, essential for ATP binding, and the GDGVND motif, involved in the hydrolysis of the acyl phosphate intermediate (Thever and Saier, 2009). The last three motifs are not completely conserved in PHA6. The 14-3-3-binding site, located at the extreme C-terminus, is absent in PHA5 and PHA6 (Supplementary Fig. S1).

Multiple alignment of the protein sequences of A. thaliana (AHA), N. plumbaginifolia (PMA), O. sativa (OSA), and S. tuberosum Phureja (PHA) PM H^+-ATPases was carried out to generate a phylogenetic tree (Fig. 1). The potato proteins were grouped into four of the five well-established subfamilies (Arango et al., 2003), except for PHA6, which does not fall into any of the subfamilies defined, and was excluded from the phylogenetic analysis. PHA1–PHA5 and PHA7 were clustered into the subfamilies I, II, IV, and V, while no members of PHAs were represented in subfamily III.

**Statistical analysis**

Statistical analysis was carried out using the Student’s t-test. A P-value <0.05 was considered statistically significantly.
Involvement of PM H⁺-ATPase in tuberization

In a first attempt to elucidate the role of PHAs in tuberization, the inhibitors of H⁺-ATPase, sodium orthovanadate (vanadate) and erythrosine B (Kanczewska et al., 2005; Hayashi et al., 2014; Yuan et al., 2017), were used in tuberization experiments in vitro. Dose–response experiments were carried out to determine the appropriate concentration of inhibitors, which were 1 mM and 50 μM for vanadate and erythrosine B, respectively (not shown). The length of inhibitor-treated stolons was drastically reduced (Fig. 3A, C). Both inhibitors increased the percentage of tuberizing stolons cultured under tuber-inducing conditions (MS medium plus 8% sucrose) (Fig. 3B, C). The tubers obtained from stolons treated with vanadate or erythrosine B were significantly smaller than those obtained from untreated stolons (Fig. 3D). When stolons were cultured under non-inducing conditions (MS medium plus 2% sucrose), inhibition of PM H⁺-ATPases by vanadate or erythrosine B also inhibited stolon growth and promoted tuber initiation (Supplementary Fig. S5).

Cloning of the PHA1 gene from S. tuberosum cv. Spunta

PHA1 was selected for further analysis. The full-length coding sequence of PHA1 was cloned from S. tuberosum cv. Spunta. The nucleotide sequence appears in the GenBank database under the accession number KX827766. This sequence is almost identical to the S. tuberosum Phureja PHA1 sequence (PGSC0003DMP400055772; Potato Genome Sequencing Consortium database), except for one nucleotide difference within the coding region, with no amino acid change. Comparing the sequence of PHA1 from S. tuberosum cv. Spunta with the sequence of PHA1 from S. tuberosum cv. Désirée (Harms et al., 1994; GenBank accession number: X76536.1), 15 nucleotide differences were found within the coding region, one of which causes an amino acid change (R170A, in Spunta).

To confirm that the PHA1 gene cloned encodes a protein with PM H⁺-ATPase activity, a functional complementation assay was carried out. The S. tuberosum cv. Spunta PHA1 coding sequence was subcloned in YEplac181 under the control of the constitutive promoter of the yeast PMA1 gene. This plasmid was introduced into the yeast strain YAK2, which is deleted from its own two PM H⁺-ATPase genes (PMA1 and PMA2), and is able to survive on galactose medium with a centromeric plasmid carrying the yeast PMA1 controlled by the GAL1 promoter (de Kerchove d’Exaerde et al., 1995). Three independent transformants (PHA1-1, PHA1-2, and PHA1-3) obtained in a galactose medium were able to sustain the growth of YAK2 yeast cells when shifted to a glucose medium (Fig. 4A), demonstrating that the PHA1 gene cloned from S. tuberosum cv. Spunta has ATPase and proton pumping activity. The same result was obtained after eliminating the plasmid bearing the yeast PM H⁺-ATPase from the YAK2-PHA1-3 strain by 5-FOA (Fig. 4B).

Overexpression of PHA1 promotes stolon growth and branching, and increases tuber weight

Transgenic plants expressing the PHA1 gene under the control of the 35S CaMV promoter (PHA1-OE) were developed.
Three independent PHA1-OE transgenic lines (L1–L3) were selected for detailed characterization. The presence of the transgene was confirmed by PCR amplification (Fig. 5A). RT–qPCR analysis showed that the mRNA abundance of PHA1 was higher in transgenic lines than in wild-type plants (Fig. 5B). Accordingly, transgenic lines exhibited a higher PM H+-ATPase activity in leaves and stolons (Fig. 5C).

After 2 weeks of culture under tuber-inducing conditions, PHA1-OE stolons were longer than wild-type stolons (Fig. 6A, B). Accordingly, transgenic stolons had longer epidermal cells (Fig. 6C, D). No branching was observed after 2 weeks (Fig. 6B). After 3 weeks of culture, the percentage of branched stolons was significantly higher in PHA1-OE stolons than in wild-type stolons (Fig. 6E, H). The primary stolon length of L1 and L3 was higher, with no statistically significant difference between L2 and wild-type stolons (Fig. 6F), although the total stolon length was increased in all three transgenic lines, due to the increased number of branches (Fig. 6G). PHA1-OE plants grown in soil presented longer stolons than wild-type plants (Fig. 7A, B), confirming the long-stolon phenotype observed in vitro.

The percentage of tuberizing stolons was not significantly affected in PHA1-OE lines (Fig. 8A); however, after 10 weeks of culture, ~26% of PHA1-OE stolons presented more than one tuber (2–5 tubers per stolon), while only 7% of wild-type stolons developed more than one tuber (Fig. 8B, C). In most cases, the additional tubers were very small or undeveloped. The tubers developed from PHA1-OE stolons were larger and presented higher starch content than those obtained from wild-type stolons (Fig. 8D–F).

The tuberization capacity of PHA1-OE plants grown in soil was determined. There were no significant differences in the number of tubers obtained per plant between PHA1-OE and the wild type (Fig. 9A). The average weight of tubers obtained from PHA1-OE plants was higher than that of wild-type tubers (Fig. 9B). The tuber yield per plant was higher in transgenic lines with respect to wild-type plants, although this difference was statistically significant only for L2 and L3 (Fig. 9C). It is important to note that the tuber yield was determined in plants transferred to soil ex vitro, which are much smaller than potato plants obtained from seed tubers, and consequently have significantly lower yields.
Overexpression of PHA1 alters cell wall invertase activity, starch content, and StSUT1 expression in stolons

Cell wall invertase activity, starch content, and StSUT1 expression were determined in stolons during the elongation stage. After 2 weeks of culture, PHA1-OE stolons showed higher cell wall invertase activity (Fig. 10A) and accumulated significantly less starch than wild-type stolons, showing very low values of starch content (Fig. 10B). Treatment with H⁺-ATPase inhibitors inhibited stolon elongation (Supplementary Fig. S6), decreased cell wall invertase activity (Fig. 10A), and increased starch accumulation (Fig. 10B) in PHA1-OE stolons, abolishing the phenotypic differences between transgenic and wild-type stolons. PHA-OE stolons

Fig. 3. Effect of vanadate and erythrosine B (EB) on stolons cultured under tuber-inducing conditions. Stolons were cultured under tuber-inducing conditions (MS medium plus 8% sucrose) in the absence (control) or presence of 1 mM vanadate or 50 μM erythrosine B. The stolon length was measured after 3 weeks (A), and the percentage of tuberizing stolons was determined at the indicated times (B). (C) Representative images of stolons. (D) Fresh weight of tubers obtained after 10 weeks of culture under tuber-inducing conditions in the absence (control) or presence of 1 mM vanadate or 50 μM erythrosine B; a representative image of the tubers obtained is shown. Quantitative data of three independent experiments (mean ± SE) are displayed in the bar graphs. The asterisks indicate statistical significance (*P<0.05, **P<0.005, ***P<0.001, with respect to control).

Fig. 4. Functional complementation of a null mutation of yeast PM H⁺-ATPase (strain YAK2) by the PHA1 gene from S. tuberosum cv. Spunta. (A) Serial dilutions (starting from an OD₆₀₀nm=1.7) of the following YAK2 yeast strains were spotted onto solid media containing glucose (MGlu-His, Leu, Trp) or galactose (MGal-His, Leu, Trp), buffered at pH 6.5: Control –, YAK2 transformed with empty YEplac181; Control +, YAK2 transformed with YEplac181-E14D, that expresses the constitutively active form of the PM H⁺-ATPase PMA2 Q42932 from N. plumbaginifolia under the yeast PMA1 promoter; PHA1ΔN, YAK2 transformed with YEplac181-PHA1ΔN that expresses a truncated, inactive form of PHA1, lacking the first 553 nucleotides of the N-terminus (devoid of the TGES motif) under the yeast PMA1 promoter; PHA1-1/2/3, YAK2 transformed with YEplac181-PHA1, that expresses PHA1 under the yeast PMA1 promoter (three different transformed yeast clones were used). (B) Serial dilutions (starting from an OD₆₀₀nm=1.7) of the following YAK2 yeast strains spotted onto solid media containing glucose (MGlu-His, Leu, Ura, Trp): Control –, PHA1–PHA3, and PHA1–PHA3 after 5-FOA treatment to eliminate the plasmid bearing the yeast PM H⁺-ATPase gene. Yeast strains were grown at 30 °C for 48 h.
Stritzler et al. showed higher expression levels of the sucrose–H⁺ symporter gene \textit{StSUT1} than wild-type stolons (Fig. 10C).

**Discussion**

The potato PM H⁺-ATPase family

An in silico screening in the \textit{S. tuberosum} Phureja genome database retrieved seven sequences, designated \textit{PHA1}–\textit{PHA7}, with a high degree of homology with PM H⁺-ATPases (Fig. 1; Supplementary Table S2; Supplementary Fig. S1), including two genes (\textit{PHA1} and \textit{PHA2}) identified earlier in \textit{S. tuberosum} cv. Désirée (Harms et al., 1994). The expression patterns of PHAs were determined in \textit{S. tuberosum} cv. Spunta. \textit{PHA1}, \textit{PHA2}, and \textit{PHA3} are ubiquitously expressed throughout the plant (Fig. 2A). These results are consistent with previous reports describing the expression patterns in other species. \textit{PHA1} and \textit{PHA3} belong to subfamily I, and \textit{PHA2} to subfamily II; the genes of these subfamilies (\textit{A. thaliana AHA1}, \textit{AHA2}, \textit{AHA3}, \textit{AHA4}, and \textit{AHA11}; tobacco \textit{PMA1}, \textit{PMA2}, \textit{PMA3}, and \textit{PMA4}; maize \textit{MHA2}; tomato \textit{LHA1}; and cucumber \textit{CsHA2}, \textit{CsHA3}, \textit{CsHA4}, \textit{CsHA8}, \textit{CsHA9}, and \textit{CsHA10}) have shown a broad expression pattern throughout the plant (Ewing and Bennett, 1994; Frías et al., 1996; Moriau et al., 1999; Oufattole et al., 2000;
Fig. 6. Phenotypic analysis of PHA1-OE stolons cultured in vitro. Stolons from wild-type (wt) and PHA1-OE plants (L) were cultured under tuber-inducing conditions (MS medium plus 8% sucrose). (A) Stolon length was determined after 2 weeks of culture; quantitative data of three independent experiments (mean ±SE) are displayed in the bar graph; a representative image is shown (B). (C) Longitudinal length of epidermal cells of the medial region of the stolons cultured for 2 weeks; the data represent the means ±SE of five biological replicates; lengths of 30–60 cells per replicate were measured. (D) Imprints of epidermal cells from the medial region of stolons, viewed by light microscopy. After 3 weeks, the percentage of branched stolons (E), primary stolon length (F), and total stolon length (G) were determined; quantitative data of three independent experiments (mean ±SE) are displayed in the bar graphs. (H) Representative images of stolons after 3 weeks of culture. The asterisks indicate statistical significance (*P<0.05, **P<0.005, ****P<0.001, with respect to the wt).

Santi et al., 2003; Lefebvre et al., 2005; Gaxiola et al., 2007; Wdowikowska and Klobus, 2016). In contrast, PHA4 and PHA5 expression is limited to flower buds and flowers, while PHA6 is only expressed in flower buds and roots (Fig. 2A), suggesting that the pumps encoded by these genes have more specialized functions. PHA4 and PHA5 belong to subfamily IV; it has been shown that the expression of genes of this subfamily is limited to floral organs: Arabidopsis AHA6 and AHA9 transcripts are present only in anthers, AHA8 and the tobacco PMA5 are expressed only in pollen, and cucumber
CsHA5, CsHA6, and CsHA7 are exclusively expressed in young flowers (Houlne and Boutry, 1994; Lefebvre et al., 2005; Gaxiola et al., 2007; Wdowikowska and Klobus, 2016). Salt stress significantly increases PHA1, PHA2, and PHA3 expression (Supplementary Fig. S4), suggesting a role for these PM H⁺-ATPases in the development of stolons and tubers. Inhibition of PM H⁺-ATPase activity by vanadate or erythrosine B inhibits stolon elongation, promotes tuber induction, and impairs tuber growth (Fig. 3; Supplementary Fig. S5), confirming that PM H⁺-ATPases are involved in tuberization acting at different stages of the process.

**PHA1 promotes stolon elongation**

PHA1 was selected for functional analysis by overexpression in potato plants. PHA1-OE stolons elongate faster and present longer epidermal cell length than wild-type stolons after 2 weeks of culture (Fig. 6A–D), indicating that PHA1 activity contributes to the stolon elongation that occurs prior to tuber initiation by promoting cell elongation. In agreement with this, inhibition of PM H⁺-ATPase activity with vanadate or erythrosine B results in shorter stolon length (Fig. 3A; Supplementary Fig. S5A). These results are consistent with the role of PM H⁺-ATPases in cell elongation and expansion described for other tissues such as hypocotyls and roots (Haruta and Sussman, 2012; Inoue et al., 2016), and Nicotiana tabacum BY-2 cells (Niczyj et al., 2016). Acidification of the apoplast by PM H⁺-ATPases leads to the wall-loosening process and cell elongation (reviewed in Hager, 2003). GA promotes stolon elongation by stimulating both cell division and cell elongation (Loy, 1977; Xu et al., 1998). PHA1 expression is induced in stolons in response to GA (Fig. 2C), thus it is possible to speculate that PHA1 mediates the GA-induced stolon elongation. However, more studies are needed to establish the mechanistic link between GA and PHA1.

**PHA1 regulates sucrose–starch metabolism in stolons**

During the stolon elongation phase, apoplastic sucrose unloading predominates (Viola et al., 2001). Although the exact mechanism occurring during this process remains unknown, sucrose can be unloaded from the phloem into the apoplast by the sucrose–H⁺ symporter StSUT1 in its inverse transport mode (Kühn et al., 2003; Carpaneto et al., 2005), or possibly by recently discovered facilitators of the SWEET family (Chen et al., 2012). The apoplastic sucrose can be converted to hexoses by a cell wall invertase, and taken up by the parenchyma cells through hexose–H⁺ symporters, not yet characterized in potato. Sucrose uptake from the apoplast might be mediated by sucrose–H⁺ symporters, possibly different from StSUT1, since it was reported that StSUT1 is localized in sieve elements but not in storage parenchyma in sink tubers (Kühn et al., 2003) (Fig. 12). Apoplastic unloading correlates with high acid invertase activity (Ross et al., 1994; Appeldoorn et al., 1997; Viola et al., 2001), and is associated with rapidly growing vegetative sink tissues (Ehness and Roitsch, 1997; Herbers and Sonnewald, 1998). During the transition from stolon to tuber, a switch from apoplastic to symplastic unloading occurs, accompanied by a switch from an invertase–sucrolytic to a sucrose synthase (SuSy)–sucrolytic pathway, leading to the starch accumulation phase and tuber growth (Viola et al., 2001) (Fig. 12).

During the elongation phase, PHA1-OE stolons grow faster (Fig. 6A), and present higher levels of cell wall invertase activity (Fig. 10A) and significantly lower starch content than wild-type stolons; moreover, starch is almost undetectable in transgenic stolons (Fig. 10B). These metabolic differences are accompanied by higher expression levels of StSUT1 in PHA1-OE stolons (Fig. 10C). In agreement with this, the PHA1-OE stolon phenotype can be reversed.
by the application of PM H\(^+\)-ATPase inhibitors, that results in shorter length, decreased cell wall invertase activity, and increased starch content (Fig. 10A, B; Supplementary Fig. S6). Based on these data, it may be hypothesized that, besides promoting stolon growth by mediating cell elongation, PHA1 regulates the sucrose–starch metabolism in elongating stolons (Fig. 12). PHA1 positively regulates cell wall invertase activity (by an unknown mechanism), and its proton pumping activity can sustain the secondary transport of hexose–H\(^+\) and sucrose–H\(^+\) symporters, promoting the uptake of hexoses and sucrose by parenchyma cells; moreover, PHA1 increases the expression of \(StSUT1\) in stolons, promoting sucrose unloading into the apoplast (Fig. 12). By doing so, PHA1 favors apoplastic unloading, sucrolysis by invertase and stolon elongation, and prevents starch accumulation, which is associated with symplastic unloading. Supporting this hypothesis, \(PHA1\) and \(StSUT1\) expression is high in wild-type stolons during the elongation phase, and decreases during tuber

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**Fig. 8.** Tuberization of PHA1-OE stolons cultured in vitro. Stolons from wild-type (wt) and PHA1-OE (L) plants were cultured under tuber-inducing conditions (MS medium plus 8% sucrose). (A) Percentage of tuberizing stolons, determined at the indicated times. (B) Percentage of stolons presenting more than one tuber after 10 weeks of culture; a representative image of the stolons is shown (C). (D) Fresh weight of tubers obtained from wild-type and PHA1-OE stolons after 10 weeks of culture. Quantitative data of three independent experiments (mean ±SE) are displayed in the bar graphs. The asterisks indicate statistical significance (*\(P<0.05\), **\(P<0.01\), ***\(P<0.005\), ****\(P<0.001\), with respect to the wt).
growth (Fig. 2B). In this context, PHA1 seems to play a key role in the molecular mechanism that determines the switch from the apoplastic unloading/invertase–sucrolytic pathway to the symplastic unloading/SuSy–sucrolytic pathway.

PHA1-OE stolons show higher expression levels of *StSUT1* (Fig. 10C), suggesting that this PM H⁺-ATPase may be part of the signaling pathway that leads to the activation of *StSUT1* transcription. It is possible that PHA1 induces the expression of *StSUT1* in companion cells, and the *StSUT1* mRNA is transported via plasmodesmata to the sieve elements as previously described (Kühn *et al.*, 1997) (Fig. 12).

**Role of PHA1 in tuber development**

PHA1-OE tubers are larger and present higher starch content than wild-type tubers (Figs 8D–F, 9B). *StSUT1* is essential for sucrose phloem loading (Kühn *et al.*, 1996); therefore, it is possible to speculate that PHA1 might enhance sucrose phloem loading by energizing this sucrose–H⁺ symporter (Fig. 12), promoting sucrose translocation to sink tubers and starch accumulation. The mechanism of sucrose phloem loading in whole plants is different from the process occurring in stolons cultured *in vitro*; however, in both cases...
sucrose loading requires a proton symport mechanism energized by PM H\(^+\)-ATPases. In plants, sucrose is loaded into the phloem in source leaves (Lemoine et al., 2013), while in stolons cultured in vitro, sucrose is obtained from the culture medium and loaded into the phloem as either hexoses (after hydrolysis by invertase) or sucrose (De Riek et al. 1997).
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PHA1-OE stolons show an increased branching phenotype (Fig. 6E, H), which results in a higher percentage of stolons presenting more than one tuber, although the additional tubers are undeveloped (Fig. 8B, C). Stolon branching increases the number of potential tuber sites; however, developing more than one tuber per stolon leads to a competition for resources that negatively affects tuber size (Celis-Gamboa et al., 2003). Interestingly, the fully developed tubers obtained from PHA1-OE stolons are larger than those from wild-type stolons (Fig. 8D, E), indicating that PHA1 positively regulates tuber growth. This result agrees with the observation that inhibition of PM H\(^+\)-ATPase activity results in smaller average tuber weight (Fig. 3D). There are no significant differences in the percentage of tuberizing stolons between PHA1-OE and wild-type plants (Fig. 8A); however, treatment with vanadate or erythrosine B enhances tuber induction (Fig. 3B; Supplementary Fig. S5B); this effect might be due to the inhibition of PHA2 or PHA3, which are also expressed in stolons, or to a non-specific action.

As observed in vitro, PHA1-OE plants grown in soil also develop larger tubers and show an increased tuber yield with respect to wild-type plants (Fig. 9B, C), suggesting that the PHA1 gene might be a potential tool to increase potato crop yield.

**Branching phenotype of PHA1-OE plants**

Stems of PHA1-OE plants grown in vitro present a highly branched phenotype, similar to PHA1-OE stolons (Fig. 11E, F; Supplementary Fig. S9). It has recently been shown that lateral bud growth depends on the amount of sucrose translocated to those buds, with sugar distribution, not auxin, being the initial regulator of apical dominance (Mason et al., 2014). It is now clear that sugar supply to axillary buds is not only essential to trigger outgrowth, but is also required to release bud dormancy (Barbier et al., 2015). Sucrose transporters and PM H\(^+\)-ATPases have been implicated in this process; in rose bush, the onset of bud outgrowth correlates with increased sugar translocation to axillary buds and the up-regulation of the sucrose–H\(^+\) symporter gene RhSUC2 (Girault et al., 2010; Henry et al., 2011); the dormancy break of Prunus persica buds is associated with sucrose uptake and PM H\(^+\)-ATPase activity (Aue et al., 1999; Maurel et al., 2004). The branched phenotype of PHA1-OE plants could be due to an increased sucrose unloading towards the lateral buds via sucrose–H\(^+\) symporters energized by PHA1; however, more studies are required to determine the role of PHA1 in axillary bud outgrowth.
PHA1 promotes plant growth

Like PHA1-OE stolons, the stems of PHA1-OE plants grown in vitro elongate faster, and show longer internodes and more leaves than wild-type plants (Fig. 11A–D). Likewise, PHA1-OE plants grown in soil are taller than wild-type plants (Fig. 11G, H). Thus, PHA1 mediates growth in the aerial part of the plant as well as in underground stolons. Accordingly, evidence has so far been sparse. It was reported that overexpression of the unmodified PM H+-ATPase gene PMA4 in tobacco has no effect on plant growth; however, the constitutive expression of a constitutively activated form of PMA4 results in abnormal leaf inclination and twisted stems, suggesting alterations in cell expansion (Gévaudant et al., 2007). Another study has shown that the Arabidopsis aha2 mutant completes its life cycle without any observable growth alteration, and the reduced-growth phenotype only becomes apparent under stress conditions that reduce the transmembrane electrical gradient and/or external proton chemical gradient (Haruta and Sussman, 2012). The present study provides strong genetic evidence for the role of PHA1 as a driver of growth in potato plants, showing a clear enhanced-growth phenotype of PHA1-OE plants.

Supplementary data

Supplementary data are available at JXB online.

Table S1. List of primers used for semi-quantitative RT–PCR.

Table S2. Chromosome localization, coding region length, predicted protein length, and molecular weight of the PHA isoforms.

Fig. S1. Alignment of the protein sequences of PHA isoforms.

Fig. S2. Quantification of RT–PCR bands of Fig. 2A.

Fig. S3. RT–PCR analysis of PHA genes in stolons.

Fig. S4. Expression of PHA genes in response to abiotic stress.

Fig. S5. Effect of vanadate and erythrosine B on stolons cultured under non-inducing conditions.

Fig. S6. Stolon length of PHA1-OE plants cultured in the presence of PM H+-ATPase inhibitors.

Fig. S7. Phenotypic analysis of PHA1-OE plants.

Fig. S8. Effect of vanadate and erythrosine B on stem length.

Fig. S9. Branching phenotype of PHA1-OE plants.

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