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ORIGINAL ARTICLE

Transcript profiling reveals that cysteine protease inhibitors are up-regulated in tuber sprouts after extended darkness

Carolina Grandellis[1,2] · Veronica Giammaria[1] · Elisa Fantino[1] · Ignacio Cerrudo[1,3,4,5] · Sandra Bachmann[1] · Franco Santin[1] · Rita M. Ulloa[1,6]

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Abstract Potato (*Solanum tuberosum* L.) tubers are an excellent staple food due to its high nutritional value. When the tuber reaches physiological competence, sprouting proceeds accompanied by changes at mRNA and protein levels. Potato tubers become a source of carbon and energy until sprouts are capable of independent growth. Transcript profiling of sprouts grown under continuous light or dark conditions was performed using the TIGR 10K EST Solanaceae microarray. The profiles analyzed show a core of highly expressed transcripts that are associated to the reactivation of growth. Under light conditions, the photosynthetic machinery was fully activated; the highest up-regulation was observed for the Rubisco activase (RCA), the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the Photosystem II 22 kDa protein (CP22) genes, among others. On the other hand, sprouts exposed to continuous darkness elongate longer, and after extended darkness, synthesis of chloroplast components was repressed, the expression of proteases was reduced while genes encoding cysteine protease inhibitors (CPIs) and metallocarboxypeptidase inhibitors (MPIs) were strongly induced. Northern blot and RT-PCR analysis confirmed that MPI levels correlated with the length of the dark period; however, CPI expression was strong only after longer periods of darkness, suggesting a feedback loop (regulation mechanism) in response to dark-induced senescence. Prevention of cysteine protease activity in etiolated sprouts exposed to extended darkness could delay senescence until they emerge to light.

Keywords *Solanum tuberosum* · Tuber sprouting · Protease inhibitors · TIGR 10K microarrays · Light/dark conditions

The experiments presented in this manuscript comply with the current laws of Argentina.

Carolina Grandellis and Veronica Giammaria contributed equally to this work.

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✉ Rita M. Ulloa
rulloa@dna.uba.ar

[1] Instituto de Investigaciones en Ingeniería Genética y Biología Molecular (INGEBI), Consejo Nacional de Investigaciones Científicas y Técnicas, Vuelta de Obligado 2490 2nd piso, C1428ADN Buenos Aires, Argentina

[2] Present address: Instituto de Biología Molecular y Celular de Rosario, Consejo Nacional de Investigaciones Científicas y Técnicas (IBR-CONICET) Ocampo y Esmeralda, Rosario, Argentina

[3] Present address: Instituto de Investigaciones Fisiológicas y Ecológicas Vinculadas a la Agricultura (IFEVA), Universidad de Buenos Aires, and Instituto de Investigaciones Biotecnológicas, Universidad Nacional de San Martín, Buenos Aires, Argentina

[4] Consejo Nacional de Investigaciones Científicas y Técnicas, Av. San Martín 4453, C1417DSE Buenos Aires, Argentina

[5] Instituto de Biotecnología (IB), Universidad Nacional de Hurlingham, Av. Vergara 2222, Villa Tesei, Hurlingham, B1688GEZ Buenos Aires, Argentina

[6] Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

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Introduction

Potato (*Solanum tuberosum* L.) plants undergo several stages during their life cycle involving stolon formation, tuberization, tuber filling, dormancy, and sprouting. This developmental sequence requires the coordinated control of physiological processes and metabolic pathways. Meristem activity plays a key role in this process, in elongating stolons and growing sprouts; the apical meristem acts as a sink for nutrients, which are sourced from the photosynthetic apparatus and tuber storage parenchyma, respectively (Viola et al. 2007).

Inactivation of the apical meristem as a result of tuber induction impacts greatly on the physiology of the entire plant. The developing tubers subsequently become the largest sinks present, accumulating starch and storage proteins (Appeldoorn 1997; Kloosterman 2008; Kloosterman et al. 2005; Visser et al. 1994; Xu et al. 1998). Starch constitutes 70–85 % of tuber dry weight and is the main reserve on which developing shoots and roots rely their nutritional supplies. The soluble proteins of potato tubers comprised primarily protease inhibitors and patatin (Pouvreau et al. 2001); the former inhibit a variety of proteases and other enzymes like invertase. Potato tubers contain five superfamilies of protease inhibitors: I3A, I13, I20, I25B, and I37 (http://www.merops.ac.uk) (Bauw et al. 2006). Inhibitors include protease inhibitors I and II (and other Ser protease inhibitors), aspartate protease inhibitors, Kunitz-type protease inhibitors, cysteine protease inhibitors, carboxypeptidase inhibitors, and multicystatin (PMC) (Pouvreau et al. 2001; Rodis and Hoff 1984).

After harvest, tubers undergo a dormancy period that varies in length according to the potato genotype and environmental conditions during tuber growth and storage. Dormancy occurs only in the tuber eyes which contain a meristem; the rest of the tuber is still metabolically active. During the rest phase, several tuber tissues undergo a functional and metabolic sink-source transition that involves structural and metabolic changes (Viola et al. 2007) and altered gene expression (Campbell et al. 2008; Ronning 2003). The completion of these transitions coincides with the reactivation of meristem functionality at dormancy break. Compositional analyses of apical buds during storage of mature tubers revealed a marked increase in carbohydrates in advance of visible bud growth showing that dormancy release is correlated with the activation of phloem unloading (Hancock et al. 2008). Once physiological competence is achieved, sprouting is controlled by the level of phytohormones: cytokinins (CK) and gibberellins (GA) are required for bud break and sprout growth, respectively (Hartmann et al. 2011); auxin seems to play a role in vascular development, while ABA and ethylene suppress tuber sprouting though the exact role of ethylene remains to be elucidated (Sonnewald and Sonnewald 2014). Control of tuber sprouting is a major objective in potato breeding; thus, numerous papers have dealt with dormancy break and with initiation of tuber sprouting (Campbell et al. 2008; Mani et al. 2014; Sonnewald and Sonnewald 2014; Liu et al. 2015).

When seed potatoes are planted in the ground, sprouts emerge and elongate to reach the soil surface. During this elongation process, the emerging sprout subsists on carbohydrates and amino acids provided by its mother tuber. This dependency can continue for some time after emergence allowing sustained growth until the plants become autotrophic. Even then, the phloem network of the mother tuber continues to transfer reserves to the haulm, probably until reserves have been exhausted. It was suggested that sprout growth could be limited by the availability of mother tuber reserves (Davies 1984). Therefore, after bud breakage, the next challenge that the sprout faces is autotrophy. In this work, our attempt was to assess the changes in gene expression which are triggered in sprouts in response to light or darkness.

The transcriptome of tuber sprouts exposed to continuous light or dark conditions for 10 or 35 days (L10, D10, D35) was analyzed using the TIGR 10K EST Solanaceae microarray. Sprouts exposed to extended dark periods (D35) adopted a skotomorphogenic program of development, in which allocation of resources was typically directed toward sprout elongation at the expense of leaf development, and solely depend on mother tuber reserves. On the contrary, sprouts emerging to light (L10) underwent a photomorphogenetic developmental process that will enable them to support their own growth as photosynthetically active plants. All the profiles analyzed show a core of highly expressed transcripts (1018) that are associated to the reactivation of growth after dormancy release. However, while light conditions resulted in a marked up-regulation of photosynthesis-related genes, a set of genes encoding cysteine protease inhibitors (CPIs) and metallocarboxypeptidase inhibitors (MPIs) were strongly induced in D35 sprouts. Expression analysis in sprouts exposed to 5, 10, or 20 days of complete darkness confirmed that MPI mRNA levels further increased with longer periods of darkness. However, CPIs showed a drastic increase only after very long periods of darkness suggesting that their expression was regulated in response to dark-induced senescence.

Experimental methods

Plant material and treatments

Experiments were performed with certified pathogen tested *Solanum tuberosum*, L. var. Spunta tubers obtained from a commercial grower (Diagnósticos Vegetales S.A., Mar del Plata, Argentina) 2 weeks after harvest. Tubers were stored in the dark at 4 or 20 °C during 8–10 weeks. Seed-tuber age was calculated from harvest, so these tubers are considered physiologically young. Tubers were in vitro established; they

were washed under tap water, incubated for 5 min in 30 % ethanol, followed by 15 min in 2 % hypochlorite solution, washed three times with sterilized distilled water, and were then cut horizontally in halves using a scalpel blade and put into sterilized flasks containing MS-agar (0.7 %).

To evaluate sprouting in dormant tubers stored at 4 °C, tuber sections were placed under complete darkness at 4 °C in MS-agar (control) or in MS-agar containing 5 μM ABA, 5 μM benzyl aminopurine (BAP), 5 μM GA_3 (Sigma-Aldrich); 0.5 mg ml^{-1} chlorocholine chloride (CCC, an inhibitor of GA synthesis), 1 % sorbitol, sucrose, or glucose; and a combination of 5 μM GA_3 and 1 % of sugar signals. Additionally, tuber sections were placed in MS-agar (control) or in MS-agar containing 5 μM GA_3 under continuous light at 20 °C. Cool white fluorescent lamps were used to achieve 300 lmol s^{-1} m^{-2}. Tuber sprouting was visually determined and a tuber was considered sprouted if it contained one or more sprouts exceeding the scale of the subtending eye scale leaves.

Another set of tubers were stored at 20 °C and put into MS-agar at 20 °C under complete darkness for 10 or 35 days (D10, D35) or under continuous light for 10 days (L10). In addition, tubers were exposed to 5 μM GA_3 for 10 days under continuous light (LGA) or for 35 days under complete darkness (DGA) and to 1 % sucrose (DS) or 1 % sucrose with 5 μM GA_3 (DSGA) for 35 days under complete darkness. The set of experiments with tubers that would be exposed to different conditions for 35 days began at day 1 while those that would be exposed for only 10 days were initiated at day 25, and all sprouts were collected at day 35. Three independent biological replicates were performed for each condition (14 half tubers in each case). At the moment of harvest, sprout number and length were measured and pictures of the different samples were taken. In all cases, the sprouts were excised, frozen in liquid nitrogen, and stored at −80 °C. Tuber sections were also exposed to dark conditions for 5, 10, or 20 days at 20 °C in MS-agar containing flasks or to continuous light for 5 or 10 days.

Microscopy techniques

Sprouts from tubers placed in darkness or continuous light for 10 days were selected randomly from each independent experiment. For light microscopy, small portions of sprout tissue (1–3 mm) were fixed overnight at 4 °C with 2.5 % (*v*/*v*) glutaraldehyde in 100 mM sodium phosphate buffer (pH 7.2). After successive ethanol dehydration steps, the samples were embedded in paraffin according to Johansen (1940). Paraffin sections (8 μm thick) were stained with toluidine blue and examined under a light microscope (Olympus DP71). For Environmental Scanning Electron Microscope (ESEM Phillips XL30) and for magnifier (Leica), fresh sprout tissues from both treatments were used.

Starch determination in potato tubers

A modified protocol from INTA (http://inta.gob.ar/documentos/test-de-degradacion-de-almidon) was used to analyze starch content in slices from sprouted and dormant tubers from *S. tuberosum* L. var Spunta. Dormant tubers were recently harvested (10 days) while sprouted tubers had been stored in the dark at 4 °C for 8 months. Tuber slices were stained during 30 s with 2-fold serial dilutions of LUGOL solution (0.16 % iodine and 0.33 % KI), then washed with water and photographed employing a Leica EZ4D magnifying glass. To estimate dry matter, dormant and sprouted tubers were cut into slices of similar size that were air-dried at room temperature for 1 week; weight was determined at 0 and 24 h and 1 week later.

RNA extraction and microarray processing

For gene expression analysis, the potato cDNA microarrays developed by TIGR (Rensink and Buell 2005) were used. Three independent biological samples with two technical replicates were analyzed for each treatment. Entire sprouts were excised and grounded under liquid nitrogen and total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. RNAs were quantified by OD: 260/280 and checked in 1 % agarose gels. Samples were adjusted to 2 μg μl^{-1} to ensure consistent labeling, frozen at −70 °C, and sent to TIGR (Potato Functional Genomics Project, Expression profiling Project Application round 9) for further procedures. The TIGR 10K EST Solanaceae microarray contained 11,412 verified cDNA clones spotted as randomized duplicates on the array. All steps of microarray processing (cDNA production, cDNA labeling, microarray hybridization, data quantification, data normalization) were carried out by the TIGR Expression Profiling Service (http://www.jcvi.org/potato/sol_ma_protocols.shtml). Hybridizations (L10 vs. D10; D35 vs. D10; DGA vs. D35; LGA vs. L10; DGA vs. DS; DSGA vs. GA; DSGA vs. DS; DSGA vs. L10) were performed using total RNA from sprouts excised from tubers exposed to the different growth conditions naming first those labeled with Cy3. The data discussed here have been deposited in NCBI's Gene expression Omnibus and are accessible through GEO series accession number GSE10492.

Transcriptome data analysis

Microarray data were normalized according to total signal and filtered, clones with zero values in any of the replicas were eliminated, and nulls and not validated spots were not considered. After filtering, 9343 clones were analyzed in L10 versus D10 array and 8696 clones in D35 versus D10 array. The Student *t* test was conducted to obtain the *p* values that were

then corrected for multiple testing using a false discovery rate (FDR) correction at 5 % using R functions (R Development Core Team 2008). Differentially expressed genes (DEGs) were identified by two criteria: (1) change in expression greater than 2-fold and (2) corrected p value ≤0.05. Expression of clones was determined as an average of M [$M = \log_2(\text{Cy3}/\text{Cy5})$] for arrays hybridized with the independent RNA isolations and the technical replicates. Clones with $M \leq -1$ or ≥ 1 were considered to be down- or up-regulated, respectively. Annotation and functional characterization were assigned using Stu_TIGR.m02 August07 (Rotter et al. 2007). A general analysis of gene expression was performed using data from L10 versus D10 and D35 versus D10 hybridizations. The other hybridizations were used to analyze the expression of protease inhibitors present in the TIGR array.

Analysis of protease inhibitors in the potato genome

Data available at http://potatogenomics.plantbiology.msu.edu/ (Potato Genome Browser v4.03) allowed us to search all the protease inhibitors present in the *Solanum phureja* potato genome and analyze their chromosome localization. The sequences of the EST clones encoding protease inhibitors in the TIGR 10K array were obtained at http://www.ncbi.nlm.nih.gov/nucest/ and blasted with SPUD DB search tools (http://potato.plantbiology.msu.edu/integrated_searches.shtml) to identify which protease inhibitor genes were represented in the TIGR array. The expression of aspartic protease inhibitors (APIs), cysteine protease inhibitors (CPIs), metallocarboxypeptidase inhibitors (MPI-1 and MPI-2), and protease inhibitors 2 (PIN2) from chromosomes 2, 3, and 11 was evaluated in the following hybridizations: L10/D10; D35/D10; DGA/D35; LGA/L10.

Phylogenetic analysis of protease inhibitor genes was performed using the protein sequences with the Neighbor-Joining method (Saitou and Nei 1987) and evolutionary analysis was conducted in MEGA5 (Tamura et al. 2013). Promoter sequences of the CPIs were analyzed using PLACE (www.dna.affrc.go.jp/PLACE/).

Expression analysis

The expression of CPIs and MPIs was evaluated in sprouts grown under dark conditions for 5, 10, or 20 days using different techniques. Total RNA (1 μg) was pre-treated with DNAse (RQ1 RNAse-free DNAse; Promega Madison, WI, USA) and reverse transcribed with M-MLV-Reverse Trancriptase (Promega) using oligo-dT primers and random hexamers (Invitrogen). Control reactions to check equal amounts of cDNA template were performed using primers EF1α Fw (5′ ATTGGAAACGGATATGCTCCA 3′) and EF1α Rev (5′ TCCTTACCTGAACGCCTGTCA 3′) to amplify a 101-bp elongation factor 1α fragment (20 cycles, annealing temperature 58 °C). Semi-quantitative RT-PCR was carried out according to Raíces et al. (2003) using primers CPI-Fw 5′ ATGATTTTGAGTTTCCTCTTGC 3′ and CPI-Rv 5′ TACATGGAGCGATAGAAGGAT 3′ (30 cycles, annealing temperature 56 °C) to amplify three of the five CPI genes, and primers MPI-Fw 5′ ACAAATAGGACATTACCTAGAA 3′ and MPI-Rv 5′ GCTTTTCTTCTGTTCATCAATG 3′ (30 cycles, annealing temperature 52 °C) to exclusively amplify MPI-1. PCRs were performed with Taq polymerase (Invitrogen). PCR products were separated and visualized in 1 % agarose gels. In addition, semi-quantitative RT-PCR assays were performed using as template total RNA from early stolons and the CPI and MPI primers.

The expression of MPI-1 was also evaluated by Northern blot and quantitative real-time PCR (qRT-PCR). Total RNA (10–20 μg) was denatured and separated on 1.4 % (*w/v*) formaldehyde agarose gels and blotted onto nylon membranes (Hybond N+; Amersham Pharmacia Biotech, Uppsala, Sweden). Northern blots were hybridized with an MPI-1 probe spanning the complete coding sequence that was labeled with dCTP [^{32}P] by random priming with the Prime-a-gene labeling system kit (Promega). Hybridization was performed overnight at 65 °C. Sequential stringent washes (2× SSC, 1× SSC, and 0.1× SSC with 0.1 % SDS) were performed at 65 °C and the blots were exposed. Signals were visualized using a STORM 830 PhosphorImager (Amersham Pharmacia Biotech) and quantified with ImageJ software. RNA loading was checked by hybridization with a labeled 18s rRNA probe. qRT-PCR was carried according to Gargantini et al. (2006). One microliter of the cDNA sample was used as template for the PCR reaction with StMPI-1 specific primers (Fw 5′ ACTGTATGTGAAACCACTGGG 3′ and Rv 5′ GCCGATTGCTTGGGAATTAC 3′). Quantification of StMPI-1 gene expression was performed using SYBR Gold and Platinum Taq polymerase® (Invitrogen) in a DNA Cycler Engine Opticom® 2 (MJ Research, Inc., MA, USA). EF1α gene was used as standard. Three independent biological replicates were used in the analysis. The real-time PCR data was generated and analyzed by the "comparative count" method to obtain relative mRNA expression of each sample as described in the Cycler manual (MJ Research).

Results

Effectors of the sprouting process

Effectors of the sprouting process were evaluated in an in vitro system using tuber half sections from young tubers (8 weeks after harvest) exposed during 25 days to complete darkness at 4 °C with the addition or not of the phytohormones BAP, ABA GA_3, or the inhibitor of GA biosynthesis CCC. As depicted in Table 1, the addition of GA_3 to the media

Table 1 The sprouting process was evaluated in an in vitro system using tuber half sections exposed to different conditions

Treatment	Days	*n*	*T* (°C)	Total number of sprouts	Sprouted eyes per tuber	Sprouts per tuber eye	Length of sprouts (mm)
Experiment 1							
D_{25}	25	4	4	19	4.2 ± 0.5	1	1
D_{25} + GA_3	25	4	4	31	7.75 ± 3.2*	2 ± 1	4.2 ± 1.3*
D_{25} + CCC	25	4	4	23	5.2 ± 0.6	1	1.5 ± 0.5
D_{25} + ABA	25	4	4	12	2 ± 1*	1	<1
D_{25} + BAP	25	4	4	21	4.5 ± 0.7	1	1.5 ± 0.5
Experiment 2							
D_{35}	35	6	4	18	3	1	<1
D_{35} + GA_3	35	6	4	42	6 ± 1*	1	5 ± 1.3*
D_{35} + sucrose	35	6	4	21	3 ± 0.5	1	<1
D_{35} + glucose	35	6	4	18	3	1	<1
D_{35} + sorbitol	35	6	4	17	3 ± 0.3	1	<1
D_{35} + sucrose + GA_3	35	6	4	45	9*	1	4.1 ± 0.8*
D_{35} + glucose + GA_3	35	6	4	44	8 ± 1.2	1	4.6 ± 1.5*
D_{35} + sorbitol + GA_3	35	6	4	37	5 ± 1.2	1	4.7 ± 1.4*
L_{10}	10	6	20	42	6 ± 1	1	14 ± 1
L_{10} + GA_3	10	6	20	43	6 ± 1	1	20 ± 2*
Experiment 3							
D_{10}	10	21	20	74	3 ± 1	1	21 ± 3
D_{35}	35	21	20	63	3	1 ± 0.2	48 ± 21
D_{35} + GA_3	35	21	20	62	2 ± 1	3 ± 1*	50 ± 10
L_{10}	10	21	20	95	4 ± 1	1	10 ± 2
L_{10} + GA_3	10	21	20	75	3 ± 1	3 ± 1*	15 ± 5*
D_{35} + sucrose	35	21	20	94	3 ± 1	1	25 ± 5
D_{35} + GA_3 + sucrose	35	21	20	84	4	3 ± 1*	30 ± 10

The length in days, temperature, number of tubers (*n*), and conditions used are indicated in each case. Numbers as subscripts indicate days of treatment. Statistical analysis of the data was performed with Student's *t* test. In each experiment, the treatments were compared to controls (D_{25}, D_{35}, and L_{10})

D dark condition, *L* light condition

*$p < 0.05$

enhanced sprout length and number, and more than one sprout emerged from each tuber eye. Sprouting was accelerated and sprout length was strongly enhanced by GA_3 when older tubers (12 weeks after harvest) were used (data not shown). On the contrary, ABA exerted an inhibitory effect on tuber sprouting.

In a similar experiment, post-harvest tubers were exposed to GA_3, sugar signals (sucrose, sorbitol, or glucose) or a combination of both GA_3 and sugars. At the moment of harvest at 35 days post-treatment (dpt), only incipient sprouts (≤1 mm) were observed in non-treated tubers while more developed sprouts (5 mm) were evident in GA-treated tubers. Besides, the addition of sugars did not affect the process (Table 1). These results are in accordance with observations described by Hartmann et al. (2011) showing that GA_3 is a sprouting-promoting factor in their one-bud in vitro system. When control and GA_3-treated tubers were exposed to continuous light conditions at 20 °C during 10 days, early sprouting was observed in both though GA_3-treated tubers were more elongated (Table 1).

In an additional experimental design, tuber sections were placed on MS media containing or not 5 μM GA_3 and/or 1 % sucrose under continuous light or dark conditions. All flasks were placed at 20 °C in order to avoid temperature effects. At 20 °C, early sprouting occurred in all conditions and sprouts had grown much more than in the previous experiments (Table 1, ESM Fig. 1). L10 and D10 sprouts were substantially different as a consequence of either photomorphogenetic or skotomorphogenetic development (ESM Fig.1a, b). On the other hand, D10 and D35 sprouts differ in their developmental

age as reflected in shoot elongation, root development, and nutrient availability.

Sprouting leads to remobilization of storage compounds, mainly starch and proteins, and shrinkage due to loss of water (Coleman 1987; Sonnewald 2001; Börnke et al. 2007). In the dark, the tuber is the only source of reserves for the developing shoots and roots; thus, the decline in reserves will be greater as the dark period extends and sprouts could suffer from carbon deprivation. In fact, tip necrosis was observed in some D35 sprouts, a sign that could indicate dark-induced senescence (ESM Fig. 1c). In this means, previous reports attribute necrosis in the sprout tip in dark grown sprouts to a localized calcium deficiency (Dostal 1943; Wien and Smith 1969) that could be related to the increasing age of the mother tuber. Starch content was analyzed in dormant tubers recently harvested and in sprouted tubers with evident signs of shrinkage (fresh weight was 116 ± 10 g and 40.3 ± 1 g in dormant and sprouted tubers, respectively). Dry matter represented 33 and 20 % of fresh weight in sprouted and dormant tubers, respectively. These tubers were selected because the etiolated sprouts were in a similar stage of development as D35 sprouts suggesting a similar metabolic situation. An evident decline in starch content was observed in the sprouted tubers (ESM Fig. 1d).

The addition of GA_3 to tubers exposed to light conditions resulted in longer and more developed sprouts (Table 1 and ESM Fig. 1f) while etiolated sprouts grown with or without GA_3 were similar in length (ESM Fig. 1c, e). However, sprout number was increased in the presence of the phytohormone under dark conditions (D35GA) and multiple sprouts emerged from each tuber eye (Table 1). When sucrose was included (D35S and D35GAS), the media was heavily contaminated and sprouts were significantly shorter than in D35 or D35GA conditions (Table 1).

Sprouts ($n = 8$) grown for 10 days under dark and light conditions were observed with a magnifier (×10). In both cases, anthocyanin pigments were detected in the sprout tips (Fig. 1). Primordial leaves were dissected and counted displaying an average of two leaves in dark grown sprouts while six leaves were observed in light grown sprouts. Light microscope analysis of paraffin-embedded sprouts confirmed the presence of well-differentiated primordial leaves with vascular tissue and epidermis surrounding the SAM and lateral buds in light grown sprouts (Fig. 1d). In contrast, dark grown sprouts were less developed; undifferentiated tissue and an epidermal layer were observed in all the buds analyzed, and only in a few cases the procambium was distinguished (data not shown). When fresh sprouts were analyzed by ESEM, differentiated leaves were evident in light grown sprouts while an ordered layer of undifferentiated cells and an incipient primordial leaf were observed in those grown in the dark (Fig. 1e).

Transcriptome analysis of light and dark grown sprouts

Transcriptome analysis of the potato sprouts grown in the latter experiment was performed using TIGR microarrays. Regardless of the culture condition, there was a core of genes (1018 clones) that were similarly expressed in L10, D10, or D35 ($p > 0.4$) correlating with the resumption of growth observed (Fig. 2a). The ratio of expression between the different conditions (D35/D10 ÷ L10/D10) was between 0.9 and 1.1 in 58.3 % of these genes, between 0.75 and 0.89 in 16.3 %, and between 1.11 and 1.25 in 19.8 %, while only 5.2 % were between 1.26 and 1.9 and 0.3 % were between 0.64 and 0.74 (clones with ratios <1 are slightly down-regulated and those with ratios >1 are slightly up-regulated under dark conditions) (Fig. 2b). These clones were classified in BIN categories according to Stu_TIGR.m02 August07 (Rotter et al. 2007). Approximately 22 % of them are related to protein metabolism (BIN 29); in particular, 53 % of the clones encoding ribosomal proteins, approximately 30 % of those involved in protein initiation, elongation, release, and targeting to nucleus, mitochondria, or secretory pathway, and 17 % of those related to protein degradation (ubiquitin pathway and proteases) have similar expression in L10, D10, or D35 conditions. Another 7.8 % is related to RNA metabolism (BIN 27), mainly associated to RNA processing and RNA binding (accounting for 11 and 12 % of the clones in these subcategories) while only 5 % of the clones associated to regulation of transcription have similar expression in the conditions analyzed. In addition, 40 % of clones with similar expression belong to BIN 35 (not assigned) and represent 8 % of the clones in this category. Other categories represented are nucleotide and DNA metabolism; transporters and proteins involved in vesicle mediated traffic; genes involved in lipid, carbohydrate, and energy metabolism; genes related to cell organization, cell cycle, and cell division; and those involved in hormone responses and signaling cascades (Fig. 2c). In particular, several proteins related to auxin signaling, cell wall metabolism, and cell cycle were actively transcribed. The expression of cell cycle genes was accompanied by active replication, as indicated by the high expression of genes such as histones H2 and H3.

In addition, D10 and D35 share 141 clones with similar expression levels ($p > 0.6$; M [$M = \log_2$(D35/D10)] between −0.24 and 0.33) that differ in L10 ($p < 0.05$) (Fig. 2a, ESM Fig. 2a). While 87 % of these genes have higher expression under light conditions ($M = \log_2$(L10/D10) between 0.32 and 1.72) and are related to protein synthesis in chloroplasts, lipid, RNA and DNA metabolism, tetrapyrrole synthesis, and cell cycle and organization, 13 % have higher expression under dark conditions ($M = \log_2$(L10/D10) between −1.05 and −0.28). In particular, one of the later clones is related to sugar signaling and nutrient physiology.

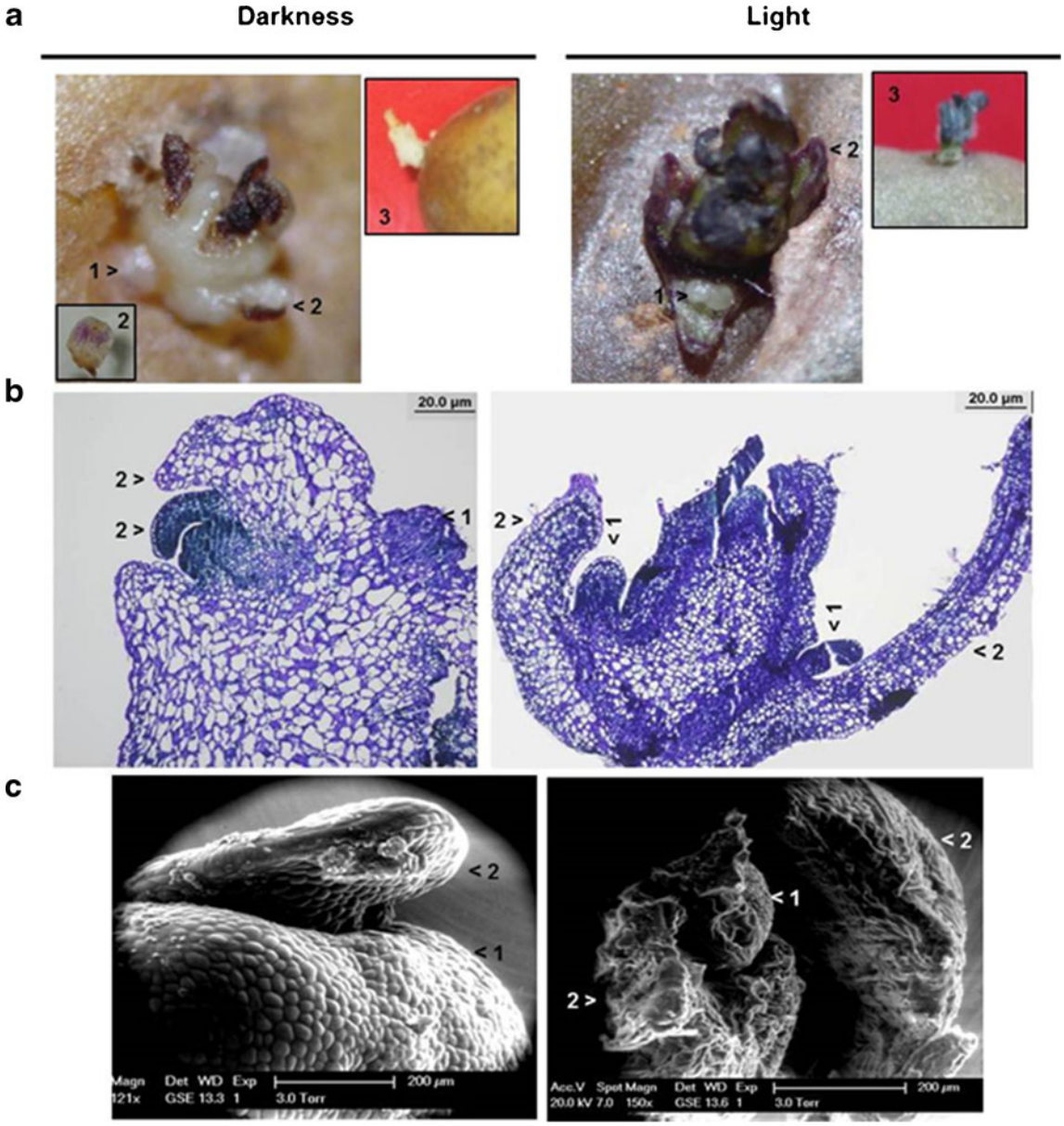


Fig. 1 Tuber sprouts exposed to dark (*left panel*) or light conditions (*right panel*) for 10 days. **a** Pictures were taken using a magnifier (×5). *Arrows* indicate sprout buds (*1*) and leaves (*2*). **b** Light microscope (×100) of 8 µm paraffin sections stained with toluidine blue. *Arrows* indicate sprout buds (*1*) and leaves (*2*). **c** Environmental scanning electron microscope (ESEM) performed using fresh sprouts. *Arrows* indicate SAM (shoot apical meristem) (*1*) and primordial sprout leaf (*2*). *Scale bars* are indicated in all cases

On the other hand, D10 and L10 sprouts share 35 clones with very similar expression levels ($p > 0.5$, $M = \log_2(\text{L10/D10})$ between −0.11 and 0.1) that have either slightly lower expression in D35 sprouts ($p < 0.4$, $M = \log_2(\text{D35/D10})$ between −0.59 and −0.23) or slightly higher expression in D35 sprouts ($p < 0.4$, $M = \log_2(\text{D35/D10})$ between 0.24 and 0.59) (ESM Fig. 2b). Seven of these clones are related to protein degradation such as cysteine proteases, SKP1-CULLIN1-F-box (SCF Box), and RING E3 ligases and ubiquitin that are down-regulated in D35 sprouts, and two E2 ubiquitin clones and two clones related to autophagy that are enhanced in D35 sprouts. Together, these transcripts indicate a tendency and suggest that protein degradation is affected under extended dark conditions.

Then we focused on those genes in L10/D10 and in D35/D10 hybridizations that were differentially expressed (DEGs) considering two criteria: (1) change in expression greater than 2-fold and (2) corrected p value ≤0.05. The differences in expression observed are related to post-sprouting processes triggered in response to the treatments applied. The 337 DEGs identified when comparing L10 versus D10 conditions represent 3.6 % of all the genes analyzed in this array and fall into 21 BIN categories (Table 2). It is known that light induces

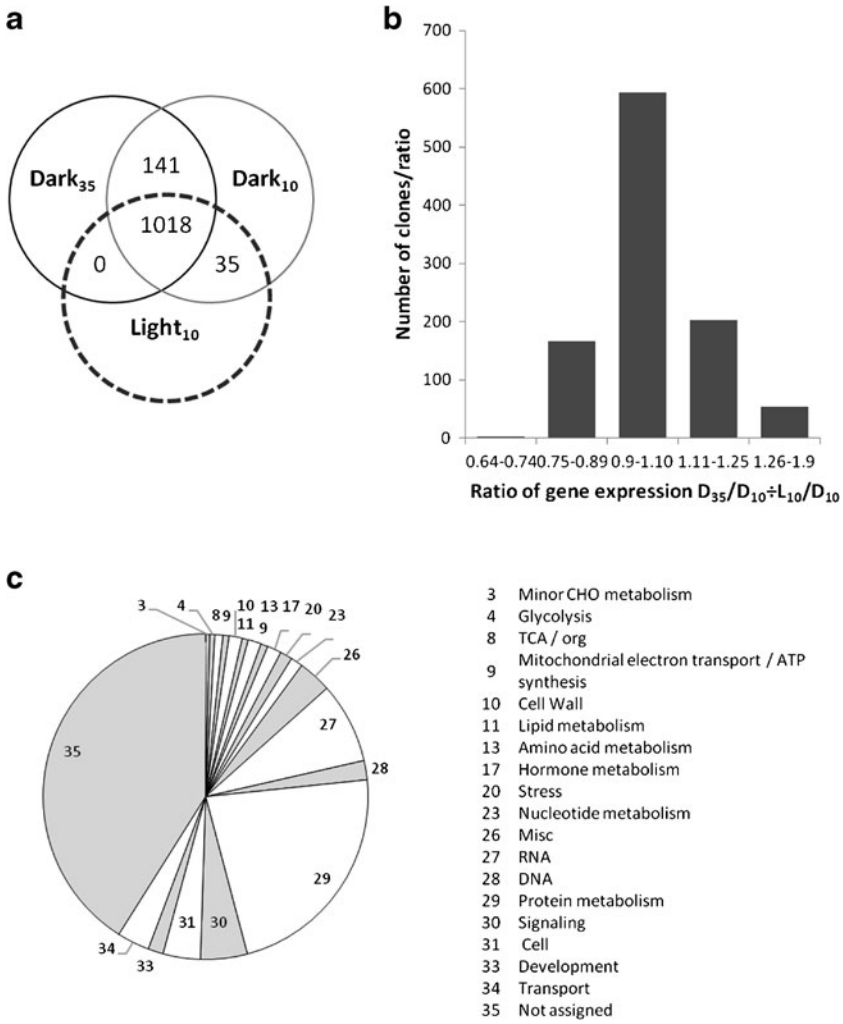


Fig. 2 Analysis of invariant genes. **a** Venn diagram showing clones with similar expression in all or in two growth conditions ($p > 0.6$). **b** Distribution of genes that have similar expression in D35 versus D10 and L10 versus D10 hybridizations according to the ratio (D35/D10 ÷ L10/D10). **c** Clones with similar expression in all conditions were grouped into functional categories (BINs) according to Stu_TIGR.m02 August07 (Rotter et al. 2007)

gene transcription and, as expected, up-regulated clones represent 92.3 % of the DEGs. In accordance with the phenotype of light grown sprouts, 27.5 % of the up-regulated clones were associated to photosynthesis (PS, BIN1). As observed in Table 2, DEGS are highly represented in light reaction (70 %) photorespiration (45 %) and Calvin cycle (54 %) subcategories. In addition, 12 % of up-regulated genes were related to protein metabolism (BIN 29) with strong representation (32 %) in the subcategory "synthesis of chloroplast components," and several up-regulated genes encode most of the enzymes involved in tetrapyrrole biosynthesis (BIN 19) ensuring coordinated chlorophyll synthesis. The constant exposure to light produces stress; in accordance with these secondary metabolites such as flavonoids and carotenoids (BIN 16), early light inducible proteins (ELIPs) and thioredoxins (BINs 20 and 21) that play a photoprotective role, as well as HSF-type transcription factors were up-regulated (BIN 27).

Under light conditions, transcription of genes encoding enzymes involved in starch and sucrose degradation was favored while expression of granule-bound starch synthase (GBSS) involved in starch synthesis was repressed (BIN2). Other up-regulated genes fall into minor CHO metabolism (BIN3), lipid, nucleotide, RNA, and DNA metabolism (BINs 11, 23, 27, and 28), development (BIN 33), and intracellular transport (BIN 34) in accordance with active growth and development (Table 2). Apart from the categories mentioned above, almost 30 % of the up-regulated clones were grouped in BIN 35 (not assigned) or BIN 26 (miscellaneous); however, when considering the number of genes in these categories, those numbers are not relevant (1.9 or 3.8 %, respectively). On the other hand, only 7.5 % of the differentially expressed clones were down-regulated under light conditions with M values close to −1. Among them are genes encoding abiotic stress proteins, such as the major latex-like protein ($M = -1.33$), germin-like proteins, and a DNAJ protein, that fall into BIN 20 and three clones encoding proline-rich cell wall proteins which represent 25 % of the clones in subBIN 10.5.2.

When the transcriptome profile of D35 sprouts was compared to that of D10 sprouts, 80 up-regulated and 88 down-regulated genes were encountered. These 168 DEGs fell into

Table 2 Differentially expressed genes (DEGs) in L10/D10 hybridization classified in BIN and SubBIN categories according to Stu_TIGR.m02 August07

L10 vs. D10 (clones in array = 9343)

BIN category	SubBIN category		Up-regulated clones = 311 (3.3 %)	Down-regulated clones = 26 (0.27 %)
1. PS (138 clones)	1.1. Light Reaction (88)		62 (70 %)	0
	1.2. Photorespiration (11)		5 (45 %)	0
	1.3. Calvin Cycle (35)		19 (54 %)	0
2. Major CHO metabolism (55)	2.1.2.synthesis.starch (19).transporter (4)		1 (transporter)	1 (starch synthase)
	2.2.1.degradation.sucrose (19).invertase (3)		1	0
	2.2.2.degradation.starch (15).transporter (3)		1	0
3. Minor CHO metabolism (52)	3.1.raffinose family.raffinose synthases.putative (2)		1	0
10. Cell walls (145)	10.5.2.cell wall proteins.proline rich proteins (12)		0	3 (25 %)
11. Lipid metabolism (179)	11.1. FA synthesis and FA elongation (53)		2 (3.8 %)	0
	11.2 FA desaturation (12)		2 (16 %)	1
	11.4.lipid degradation.lipases (17)		0	1
13. Amino acid metabolism (162)	13.2.2. degradation.glutamate family (10)		1	0
16. Secondary metabolism (168)	16.1. isoprenoids (50)		3	0
	16.4.N misc (11)		1	0
	16.8.flavonoids (38) 9 (24 %)		0	1
17. Hormone metabolism (181)	17.2. Auxin.induced (63)		1	1
	17.5. Ethylene (34)		1 (synthesis-degradation)	1 (signal transduction)
	17.7.JA.synthesis-degradation (12)		1 (lipoxygenase)	1 (12-Oxo-PDA-reductase)
19. Tetrapyrrole synthesis (33)	19.1.glu-tRNA synthetase (4)		1	0
	19.5.porphobilinogen deaminase (2)		1	0
	19.7.uroporphyrinogen decarboxylase (2)		1	0
	19.10.magnesium chelatase (3)		1	0
	19.11.magnesium protoporphyrin IX methyltransferase (1)		1	0
	19.12.magnesium-protoporphyrin IX monomethyl ester (oxidative) cyclase (2)		2	0
	19.14.protochlorophyllide reductase (2)		2	0
	19.16.chlorophyll synthase (2)		1	0
20.1. Stress. Biotic (158)	20.1.8.secondary metabolites (2)		1	0
20.2. Stress. Abiotic (167)	20.2.1. Heat (77)		2	1
	20.2.3.drought/salt (32)		2	0
	20.2.5.light (7)		3 (42 %)	0
	20.2.99.unspecified (29)		4	4
21. Redox (98)	21.1.redox.thioredoxin (38)		1	0
23. Nucleotide Metabolism (80)	23.3.salvage (11)		2	0
26. Miscellaneous (387)	26.2. UDP glucosyl and glucoronyl transferases (57)		1	1
	26.3.gluco-, galacto- and mannosidases (14)		1	0
	26.6.O- methyl transferases (3)		1	0
	26.10.cytochrome P450 (70)		4	0
	26.13.acid and other phosphatases (18)		0	1
	26.16.myrosinases-lectin-jacalin (15)		0	1
	26.18.invertase/pectin methylesterase inhibitor family protein (12)		1	0
	26.21.protease inhibitor/seed storage/lipid transfer protein (LTP) family protein (20)		1	1
	26.22.short chain dehydrogenase/reductase (24)		1	0
	24.23.rhodanese (4)		1	0
	26.28. GDSL-motif lipase (21)		4	1
27. RNA (780)	27.1.processing (96)		1	0
	27.2. Transcription (31)		2	0
	27.3.regulation of transcription (626)	27.3.6.bHLH family (41)	3	0
		27.3.23. HSF, Heat-shock TF family (3)	**2**	0
		27.3.25. MYB domain TF family (35)	1	0
		27.3.41.B3 TF family (5)	1	0
	27.3.99.unclassified (115)		3	0
28. DNA (130)	28.1.synthesis/chromatin structure (97)		3	0
29. Protein (1179)	29.1.aa activation (30)		0	0

Table 2 (continued)

L10 vs. D10 (clones in array = 9343)

BIN category	SubBIN category	Up-regulated clones = 311 (3.3 %)	Down-regulated clones = 26 (0.27 %)
	29.2.1.synthesis.chloroplast/mito - plastid ribosomal protein (43)	14 (32 %)	0
	29.2.2.synthesis.misc ribosomal protein (165)	4	0
	29.2.3.synthesis.initiation (45)	0	0
	29.2.4.synthesis.elongation (26)	1	0
	29.2.5.synthesis.release (6)	0	0
	29.3.1.targeting.nucleus (19)	1	0
	29.3.2.targeting.mitochondria (12)	0	0
	29.3.3.targeting.chloroplast (6)	1	0
	29.3.4.99.targeting.secretory pathway.unspecified (36)	1	0
	29.4.postranslational modification (218)	3	0
	29.5.degradation (55)	2	0
	29.5.1.degradation.subtilases (15)	2	0
	29.5.7.degradation.metalloprotease (16)	1	0
	29.5.11.degradation.ubiquitin (317)	3	0
	29.5.15.protein degradation.inhibitors (25)	4	0
	29.6.folding (19)	4	0
	29.8 assembly and cofactor ligation (14)	2	0
30. Signaling (319)	30.2.8.receptor kinases.leucine rich repeat VIII-1 (60)	1	0
	30.5.G-proteins (75)	1	0
31. Cell (211)	31.1.organization (97)	1	0
	31.2.division (25)	1	0
	31.3.cycle (36)	0	0
	31.4.vesicle transport (53)	0	0
33. Development (107)	33.1.storage proteins (16)	3 (18 %)	0
	33.99.unspecified (84)	8 (9.5 %)	0
34. Transport (225)	34.14.unspecified cations (24)	2	0
	34.16. ABC transporters and multidrug resistance systems (22)	2	0
	34.20.porins (7)	1	0
	34.22.cyclic nucleotide or calcium regulated channels (6)	1	0
	34.99.misc (26)	1	0
35. Not assigned (4104)	35.1.no ontology (1133)	21 (1.8 %)	3 (0.26 %)
	35.2.unknown (2971)	58 (1.95 %)	5 (0.17 %)

Numbers in brackets indicate total clones in the BIN category or subcategory. % indicates DEGs/clones in category

20 BIN categories (Table 3). Most (35 %) of the strongly repressed genes in etiolated D35 sprouts were associated to photosynthesis (BIN 1), in particular 16.4 % of the clones belonging to light reaction subcategory and 32 % of those belonging to Calvin cycle subcategory (Table 3). On the other hand, the strongly up-regulated genes in D35 sprouts represent 41 % of the clones in the subcategory inhibition of protein degradation (Table 3).

Differential expression in L10, D10, and D35 sprouts was related to photosynthesis, cell wall metabolism, and protein metabolism

As mentioned, the expression of proteins involved in light reactions, photorespiration, and Calvin cycle (BIN 1) was strongly enhanced in light compared to dark grown sprouts (Table 2, Fig. 3a). The highest up-regulation was observed for the ribulose-1,5-bisphosphate carboxylase/oxygenase activase (RCA) and the glyceraldehyde-3-phosphate dehydrogenase (GPDH) genes ($M > 3$ in L10/D10). The ferredoxin-thioredoxin system, the nuclear encoded small subunits of RuBisCO, and the ATP synthase chains δ and γ were also highly induced ($M \geq 2$). The expression of RCA, GPDH, OEE1, and ATP synthase chains δ and γ was similar in D10 and D35 sprouts; on the contrary, transcription of different RuBisCO small chain subunits, chlorophyll binding proteins, light harvesting proteins from photosystems I and II, and the ATP synthase β subunit from chloroplasts was repressed after an extended dark period ($M < 1$ in D35/D10) (Fig. 3b). However, the addition of GA to the media in dark grown sprouts (GAD) significantly enhanced the expression of 15 clones encoding RuBisCO small chain subunits in GAD versus D35 hybridization (data not shown).

As shown in ESM Fig. 1, D35 sprouts exhibited excessive shoot elongation and root development compared to D10 sprouts. Differential expression of genes related to cell wall

Table 3 Differentially expressed genes (DEGs) in D35 /D10 hybridization classified in BIN and SubBIN categories according to Stu_TIGR.m02 August07 (numbers in brackets indicate total clones in the BIN category or subcategory; % indicates DEGs/clones in category)

D35 vs. D10 (clones in array = 8695)			
BIN category	SubBIN category	Up-regulated clones = 80 (0.92 %)	Down-regulated clones = 88 (1 %)
0. Control genes (22 clones)	0. PSII type I CAB	0	1
1. PS (145 clones)	1.1.lightreaction (85)	0	14 (16.4 %)
	1.2.3.aminotransferases peroxisomal (3)	1	0
	1.3.2.calvin cycle (47).rubisco small subunit (21)	0	15 (32 %)
	1.3.7.calvin cycle (47). FBPase (3)	0	1
	2.1.synthesis (19).2.starch (17).2.starch synthase (7)	1	0
10. Cell wall (144 clones)	10.5.cell wall proteins (26).1. AGPs (5)	0	1
	10.6.degradation (49).1.cellulases and beta −1,4-glucanases (10)	0	1
	10.6.degradation (49).2.mannan-xylose-arabinose-fucose (18)	0	1
	10.6.degradation (49).3.pectate lyases and polygalacturonases (21)	0	1
	10.7.modification (14)	2	1
	10.8.pectin*esterases (13) 99.misc (5)	0	1
11. Lipid metabolism (168 clones)	11.6.lipid transfer proteins (3)	0	1
	11.9.lipid degradation (52).3.lysophospholipases (13)	0	1
12. N-metabolism (12 clones)	12.2.1.ammonia metabolism.glutamate synthase (4)	0	1
15. Metal handling (21 clones)	15.2.binding, chelation and storage (16)	1	0
16. Secondary metabolism (151 clones)	16.1.isoprenoids (44).4.carotenoids (10)	1	0
	16.10.simple phenols (10)	1	0
17. Hormone metabolism (169)	17.5.ethylene (30).1.synthesis-degradation (11)	0	1
19. Tetrapyrrole synthesis (29 clones)	19.14.protochlorophyllide reductase (2)	0	3
20.1. Stress. Biotic (158)	20.1.biotic (141).2.receptors (56)	0	1
	20.1.biotic (141).7.PR-proteins (31)	0	0
20.2. Stress. Abiotic (167)	20.2.abiotic (162).99.unspecified (29)	3	2
	20.2.abiotic (10)	1	0
	20.(10)	1	0
21. Redox (88 clones)	21.1.thioredoxin (36)	2	1
	21.3.heme (1)	0	1
	23.3.salvage (9).1.phosphoribosyltransferases (5).3.upp (3)	0	1
23. Nucleotide metabolism (74 clones)	23.4.99.phosphotransfer and pyrophosphatases.misc (21)	1	0
	26.2. UDP glucosyl and glucoronyl transferases (54)	1	0
26. Miscellaneous (356 clones)	26.7.oxidases - copper, flavone etc.(21)	0	1
	26.10.cytochrome P450 (65)	0	1
	26.12.peroxidases (26)	0	1
	26.13.acid and other phosphatases (16)	0	1
	26.21.protease inhibitor/seed storage/lipid transfer protein (LTP) family protein (20)	2	4
	26.23.rhodanese (2)	0	1
	27.1.processing (91)	1	0
27. RNA (748 clones)	27.3.regulation of transcription (596).25. MYB domain transcription factor family (35)	1	1
	27.3.regulation of transcription (596).66. Pseudo ARR transcription factor family (5)	0	1
	29.3.2.targeting.mitochondria (12)	1	0
29. Protein (1115 clones)	29.3.4.targeting.secretory pathway (62).3.vacuole (19)	1	0
	29.4.postranslational modification (203)	1	1
	29.5.degradation (472).1.subtilases (13)	0	3
	29.5.degradation (472).3.cysteine protease (25)	0	1

Table 3 (continued)

D35 vs. D10 (clones in array = 8695)			
BIN category	SubBIN category	Up-regulated clones = 80 (0.92 %)	Down-regulated clones = 88 (1 %)
	29.5.2.degradation (472).autophagy (10)	1	0
	29.5.11.degradation (472).ubiquitin (31)	1	0
	29.5.15.protein degradation.inhibitors (22)	9 (41 %)	0
30. Signaling (295 clones)	30.2.receptor kinases (92).8.leucine rich repeat (55). VIII-2 (6)	1	0
31. Cell (145)	31.1.organisation (85)	0	4
33. Development (103)	33.1.storage proteins (15)	0	2
	33.99.unspecified (80)	0	2
34. Transport (214 clones)	34.1.p- and v-ATPases (22)	1	0
35. Not assigned (3753)	35.1.no ontology (1064)	8	3
	35.2.unknown (2689)	37 (1.37 %)	11 (0.4 %)s

metabolism (BIN10) and to ethylene synthesis (BIN 17) supports the observed sprout elongation. Several clones encoding cell wall proteins (HPRG) and the brassinosteroid regulated protein (BRU1) are up-regulated under extended darkness (D35) (Fig. 3c). BRU1 is homologous to a xyloglucan endotransglycosylase, and it was reported that it has a possible role in brassinosteroid-stimulated elongation of soybean epicotyls (Zurek and Clouse 1994). On the other hand, several cell wall degrading enzymes were down-regulated in D35 sprouts. In addition, a clone encoding ACC synthase was strongly repressed ($M = -4.24$); therefore, ethylene levels which negatively regulate hypocotyl and root elongation (Muday et al. 2012) were probably low.

When analyzing protein metabolism, genes encoding chloroplast ribosomal proteins, genes related to protein targeting to chloroplasts, protein folding, and protein assembly in chloroplasts were up-regulated under light conditions (Table 2). On the other hand, six genes related to protein degradation (metalloprotease, peptidase, and ubiquitin) were induced as well. When comparing D10 and D35 sprouts, there was no difference regarding protein synthesis; however, genes involved in protein degradation and in inhibition of protein degradation were differentially expressed (Fig. 3d). In sprouts exposed to a very long dark period (D35), transcription of serine and cysteine proteases was down-regulated while several clones encoding cysteine protease inhibitors (STMCE08, STMCL26, STMCV60, STMCX33, and STMHQ67, CPIs), metallocarboxypeptidase inhibitors (STMCG57, STMGL75, MPIs), an aspartic protease inhibitor (STMCQ55), and clone STMDB78 encoding miraculin were strikingly up-regulated (Fig. 3d). The CPI clones are similar in sequence (ESM Fig. 3a) and share high sequence homology (>70 %) with transcripts PGSC0003DMT400026265, 400026272, 400026276, and 400026285, encoding CPIs type 1 and PGSC0003DMT400026288 encoding CPI type 9 (ESM Fig. 3b).

Protease inhibitors in the *Solanum phureja* genome

Potato tuber protease inhibitors are a diverse group of proteins that inhibit a variety of proteases and some other enzymes, such as invertase. Some have dual or broad substrate specificity, and they differ in their amino acid sequence, chain length, and subunit composition. Out of the 39,031 genes reported in the genome of *Solanum phureja* (http://potatogenomics.plantbiology.msu.edu/), 63 genes correspond to protease or proteinase inhibitors (confirmed by BlastX). According to the database, these genes were identified as aspartic protease inhibitors (type 5, 8, and 10), cysteine protease inhibitors (type 1, 9, 5, and B) and cysteine proteinase inhibitors, ethylene responsive proteinase inhibitor 1, inter-alpha-trypsin inhibitor heavy chain, Kunitz-type inhibitors, metallocarboxypeptidase inhibitors, proteinase inhibitor type-2 (PI-2), protease inhibitor/seed storage/lipid transfer protein family protein (LTP), serine protease inhibitors (five type I, one 7 and 3 Kazal-type family protein), serine-type endopeptidase inhibitor, trypsin inhibitors, protease inhibitors I, IIa, 14, II, or IB, and miraculin.

These protease inhibitor genes are distributed in the 12 chromosomes of *S. phureja*; however, 27 map in chromosome 3 and 20 of them are clustered in a region comprised between 49.48 and 50.47 Mb. Among them, the five CPIs (four type 1, one type 9) are located at positions 49498.1, 49548.3, 49644.5, 49685.4, and 49784.9 kb (Fig. 4a, the asterisk indicates those genes present in the array) and almost all PIN-2. Phylogenetic analysis of the inhibitors that map in chromosome 3 together with the MPIs that map in chromosome 7 and two other PIN-2 inhibitors that are located in chromosomes 2

and 11 was inferred using the Neighbor-Joining method (Saitou and Nei 1987), and evolutionary analysis was conducted in MEGA5 (Tamura et al. 2011). The phylogenetic tree shows that the five CPIs cluster together with a Kunitz-type invertase inhibitor (KII), and the four MPIs compose a different group as well as all the PIN-2 inhibitors (Fig. 4b). Sequence identity is high (87 to 97 %) among CPIs as observed in ESM Fig. 3c.

In addition to CPIs and MPIs, other protease inhibitors are represented in the TIGR 10K array such as aspartic PIs: API (clones STMCQ07, STMCQ55) and PIN2: (STMIY57, STMCP16). The expression of these inhibitors was analyzed in the different hybridizations (L10 vs. D10; D35 vs. D10; DGA vs. D35; LGA vs. L10) considering all the values from the different clones (Fig. 5). CPIs presented the highest expression levels compared to the other protease inhibitors. As observed in Fig. 5a, CPIs were significantly induced ($M=4$, $p<0.001$) under extended dark conditions (D35/D10). A significant increase in CPIs was also observed in sprouts grown under extended dark conditions with sucrose and GA in the media compared to those grown under light conditions (data not shown). Since the expression of CPIs was higher in L10 sprouts than in D10 sprouts, probably darkness per se was not the cause of the induction observed in D35 sprouts. Regarding MPI transcripts (Fig. 5c), expression was also induced under extended dark conditions (D35/D10) and was slightly lower in light conditions (D10/L10). On the other hand, APIs were similarly expressed in dark or light conditions (Fig. 5b) while the expression of PIN-2 inhibitors was higher under light conditions (Fig. 5d). It is noteworthy that the addition of GA under dark conditions reduced the expression of most inhibitors while it enhanced the expression of serine proteases ($M=1.7$).

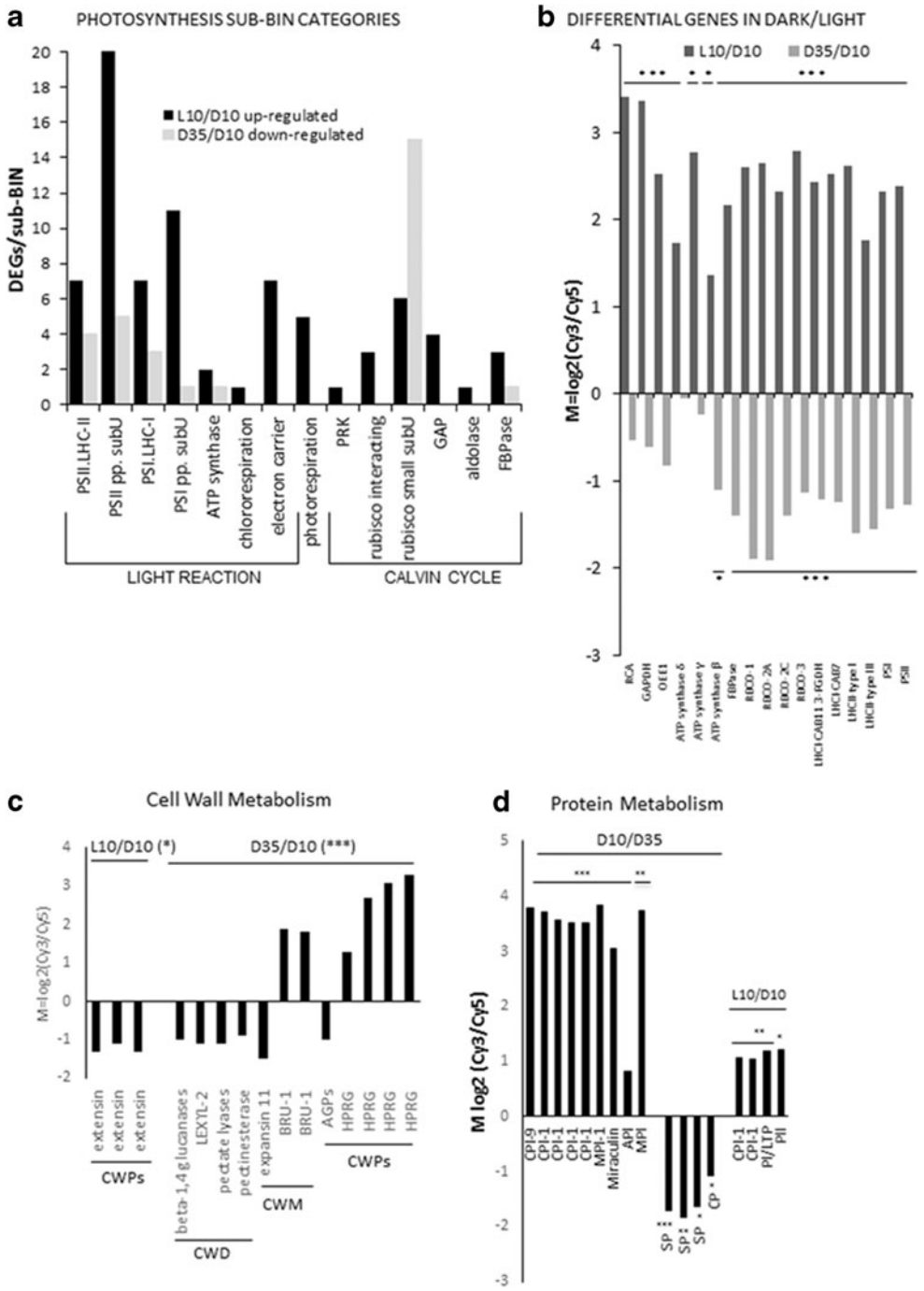
Cloning of CPIs and MPIs was performed using RNA from D35 sprouts from Spunta cv. as template: the CPI amplicon shared 92.2, 90.69, 90.49, 91.48, and 89.07 % homology with transcripts PGSC0003DMT400026272, 400026285, 400026289, P400026276, and 400026265, encoding CPIs. The MPI amplicon was 99.81 % homologous to transcript PGSC0003DMT400001813 and 98.09 % to transcript PGSC0003DMT400081485. To analyze if the increase observed in PIs expression was a consequence of extended darkness, sprouting tubers were exposed during 5, 10, or 20 days to continuous darkness. As observed in Fig. 6a, CPI expression was very similar in sprouts exposed to continuous darkness for 5 or 10 days; however, a significant increase was observed in sprouts exposed to 20 days of darkness. It should be noted that the primers used in this experiment were able to amplify transcripts 26272, 26285, and 26289. The expression of MPIs was analyzed by semi-quantitative RT-PCR, qRT-PCR, and Northern blot (Fig. 6b, c, d). As observed, the expression of MPI increased in correlation with the length of the dark period. In RT-PCR assays performed with the CPI and MPI primers, we detected the transcripts in developing stolons (data not shown).

Tubers have massive starch reserves in order to sustain growth of the developing sprouts until these reach the soil surface. However, an evident decline in starch content was observed in sprouted tubers (ESM Fig. 1d). The huge increase in the expression of CPIs in D35 sprouts could be a consequence of dark-induced senescence due to carbon starvation. An in silico search for DNA elements in the promoters of CPIs genes was performed, and the data revealed that they contain ABRELATERD1 consensus sequence ACGTG for ABRE-like sequence and MYCATERD1 consensus sequence CATGTG which have been previously described as induced in dark senescence and during dehydration stress (Simpson et al. 2003). The gene PGSC0003DMG400010134 contains the ACGTG element at position −125 from the ATG, PGSC0003DMG400010137 contains ACGTG −882 and CATGTG at −2924, PGSC0003DMG400010139 contains CATGTG at −2788, PGSC0003DMG400010143 possesses the sequence ACGTG at −2277, and finally PGSC0003DMG400010145 contains ACGTG at position −2291, as depicted in Fig. 7.

Discussion

The sprouting process is governed by phytohormones and external stimuli (Sonnewald and Sonnewald 2014); we confirmed the promoting effect of high temperature, wounding (cutting the tubers in halves) and GA, and the inhibitory effect of ABA (Table 1) on tuber sprouting and observed that the addition of exogenous sugars did not affect the process. Our findings indicate that sprouting triggered by exogenously applied GA was age dependent; it was more premature and robust in aged tubers (12 weeks post-harvest) compared to 8-week post-harvest tubers. GAs control many aspects of plant growth such as stem elongation, seed germination, and flower and fruit development (Gupta et al. 2015). The exposure of tuber halve sections to GA resulted in longer sprouts compared to controls, indicative of a role for GA on promoting cell elongation during sprouting. Supporting this GA effect, tubers exposed for 10 days to GA in light conditions at 20 °C developed prominently elongated sprouts compared to the controls with no additives. However, under extended dark conditions (35 days), the addition of GA did not result in longer sprouts but in more sprouts emerging from one tuber eye.

At the onset of sprouting, tubers switch from import (sink stage) to export (source stage) of carbohydrates, amino acids, and other compounds. These events are influenced by transcriptome changes that rearrange the developmental fate of the tuber. TIGR microarrays analysis allowed us to conduct an outline of the potato transcriptome during sprouting. The



transcriptome profile revealed 1018 clones similarly expressed in L10, D10, or D35 conditions. These clones are related to protein, RNA, nucleotide and DNA metabolism, transporters, vesicle trafficking proteins, lipid, carbohydrate and energy metabolism, cell organization, cell cycle and cell division, and genes involved in hormone responses and signaling cascades. Particularly, several auxin signaling effectors, cell wall, and cell cycle related proteins were expressed. Liu et al. 2015 reported that auxin genes were overexpressed in dormant tubers versus dormancy released tubers and found that histone and cyclin isoforms associated genes involved in cell division/cycle were up-regulated in dormant tubers versus dormancy released tubers. We also observed auxin-dependent gene expression and high expression of histone H2 and H3 once sprouting is triggered.

During dark conditions, sprouts express genes related to sugar signaling and ethylene response. The expression of sugar signaling effectors is in accordance with previous studies showing that sucrose availability is one prerequisite for bud break, and sucrose is likely to serve as nutrient and signal

◀**Fig. 3** Comparison of DEGs related to photosynthesis (**a**, **b**), cell wall metabolism (**c**), and protein metabolism (**d**) in L10/D10 and D35/D10 hybridizations. **a** DEGs were grouped according to the photosynthesis subBIN categories. *Dark gray bars* represent up-regulated genes under light conditions (L10) while *light gray bars* represent down-regulated genes under extended darkness (D35). **b** *M* values ($M = \log_2(Cy3/Cy5)$) for L10/D10 (*dark gray bars*) or D35/D10 (*light gray bars*) were estimated using the data from several clones that correspond to the same Unigene. RCA, ribulose-1,5-bisphosphatecarboxylase/oxygenase (RuBisCO) activase gene; GPDH, glyceraldehyde-3-phosphate dehydrogenase; OEE1, oxygen evolving enhancer 1; FBPase, fructose 1,6-biphosphatase; RBCO-1, RuBisCO small chain 1; RBCO-2A, RuBisCO small chain 2A; RBCO-2C, RuBisCO small chain 2C; RBCO-3, RuBisCO small chain 3; LHCI CAB 11, chlorophyll a/b-binding protein (cab-11) D-3-phosphoglycerate dehydrogenase; LHC1-CAB7, chlorophyll a-b binding protein 7 chloroplast precursor (LHCI type II CAB-7); LHCII type I, chlorophyll a-b binding protein 1B, chloroplast precursor; LHCII type III, chlorophyll A-B binding protein/(LHCB3) chloroplast precursor; PSI, Photosystem I reaction center subunit PSI-N; PSII, Photosystem II reaction center W protein chloroplast. **c** M [$M = \log_2(Cy3/Cy5)$] values of DEGs associated to cell wall metabolism in D10 versus L10 or in D35 versus D10 hybridizations are plotted. Genes were grouped according to subBIN categories. CWPs, cell wall proteins; CWD, cell wall degradation; CWM, cell wall modification. **d** M [$M = \log_2(Cy3/Cy5)$] values of DEGs associated to protein metabolism in D10 versus L10 or in D35 versus D10 hybridization are plotted. Genes are related to protein degradation (PD) or inhibition of protein degradation (IPD) categories. SP, serine protease; CP, cysteine protease; CPI, cysteine protease inhibitor; MPI, metallocarboxypeptidase inhibitor; API, aspartate protease inhibitor. The *M* values plotted correspond to genes that are differentially expressed in light versus dark (L10/D10) conditions or in extended darkness versus dark conditions (D35/D10) with $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.005$ (***) as indicated

molecule at the same time (Sonnewald and Sonnewald 2014). Besides, transition from dormancy to active sprouting is accompanied by carbohydrate metabolism (Sreenivasulu et al. 2008; Liu et al. 2015). Both ABA and ethylene suppress tuber sprouting; however, the exact role of ethylene remains to be elucidated (Mani et al. 2014). Our data indicate that ethylene response genes are expressed in the dark and not in light conditions, which accelerates sprouting.

As expected, the outcome of L10 versus D10 hybridization is coincident with a photomorphogenetic versus skotomorphogenetic developmental program where most up-regulated genes are associated to photosynthesis. The ferredoxin-thioredoxin system which provides a mechanism for light-dependent activation of RCA, GAPDH, and ATP synthase, ensuring that carbohydrate synthesis proceeds in the light, was induced differentially in response to light in L10 sprouts. These findings correlate well with a previous report showing that the RCA gene contains DNA elements that confer promoter activity with organ-specific and light-inducible features (Elmore et al. 2012; Qu et al. 2011). The thioredoxin system enhances translation and fatty acid biosynthesis and inhibits catabolic processes, altogether providing energy and promoting the observed sprout development. Experimental evidence in wheat indicates that light-mediated gene regulation may involve the Nuclear Factor Y (NF-Y) proteins (Stephenson et al. 2011). TaNF-YB3 is involved in the positive regulation of a number of photosynthesis genes by binding to light-responsive CCAAT-box cis-elements in their promoters. A likely candidate to play this role in potato is clone STMDQ49 that encodes a CCAAT-box binding transcription factor subunit B (NF-YB) (HAP3). This clone was differentially expressed in L10 versus D10 conditions ($p = 0.02197$, cy3/cy5 = 1.93, $M = 0.95$) correlating well with the expression of the genes mentioned above.

ROS species production is enhanced during active photosynthesis; therefore, plants had to evolve different strategies to cope with these potentially toxic compounds (Mittler 2002). Transcription factors of the WRKY family and HSF were induced in *Arabidopsis thaliana* plants exposed to methyl viologen that produces the superoxide anion$O^{2\bullet-}$ in the light. The promoters of genes highly up-regulated by superoxide contained the W-box consensus sequence of WRKY transcription factors (Scarpeci et al. 2008). In sprouts exposed to continuous light, clone STMIW46 encoding a WRKY-type DNA binding protein was induced 4-fold ($M = 2.04$) and clone STMEZ38 encoding an HSF-type DNA binding domain was induced 2-fold ($M = 1.15$); these transcription factors might be involved in a similar antioxidant response in illuminated potato sprouts.

Usually, once sprouts emerge and rooting begins, the plant develops leaves and is capable to support its own growth due to photosynthesis. However, carbohydrate deprivation is a fact of life for higher plants. Plants may experience carbon starvation when the emergence of young seedlings and their transition to autotrophy is delayed (Elamrani et al. 1994), when competition occurs between sink tissues such as roots, flowers, or fruits (Dejong and Grossman 1995; Ho 1996), or when under environmental constraints, the photosynthesis rate or the translocation of nutrients to sink tissues decreases (Amthor and McCree 1990; Setter et al. 1979). Similarly, in some types of senescence (Noodén 1988) or in post-harvest situations (King et al. 1990), the degradation of some tissue or cell structures is clearly related to the appearance of carbon-starvation symptoms particularly in sink organs with active metabolism (Baysdorfer et al. 1988; Thomas 1978). To survive, plant cells have to adapt to the lack of carbohydrates by substituting protein and lipid metabolism for sugar metabolism through autophagic processes (reviewed in Brouquisse et al. 1998).

D10 and L10 sprouts share 33 clones with similar expression such as cysteine proteases (CPs), SCF Box and RING E3 ligases, and ubiquitin genes, all related to protein degradation. In sprouts exposed to an extended period of darkness (D35), that solely depend on tuber reserves, changes in the expression of genes involved in protein degradation and in inhibition of protein degradation were evident: serine and cysteine proteases were down-regulated and CPIs or MPIs were strongly up-

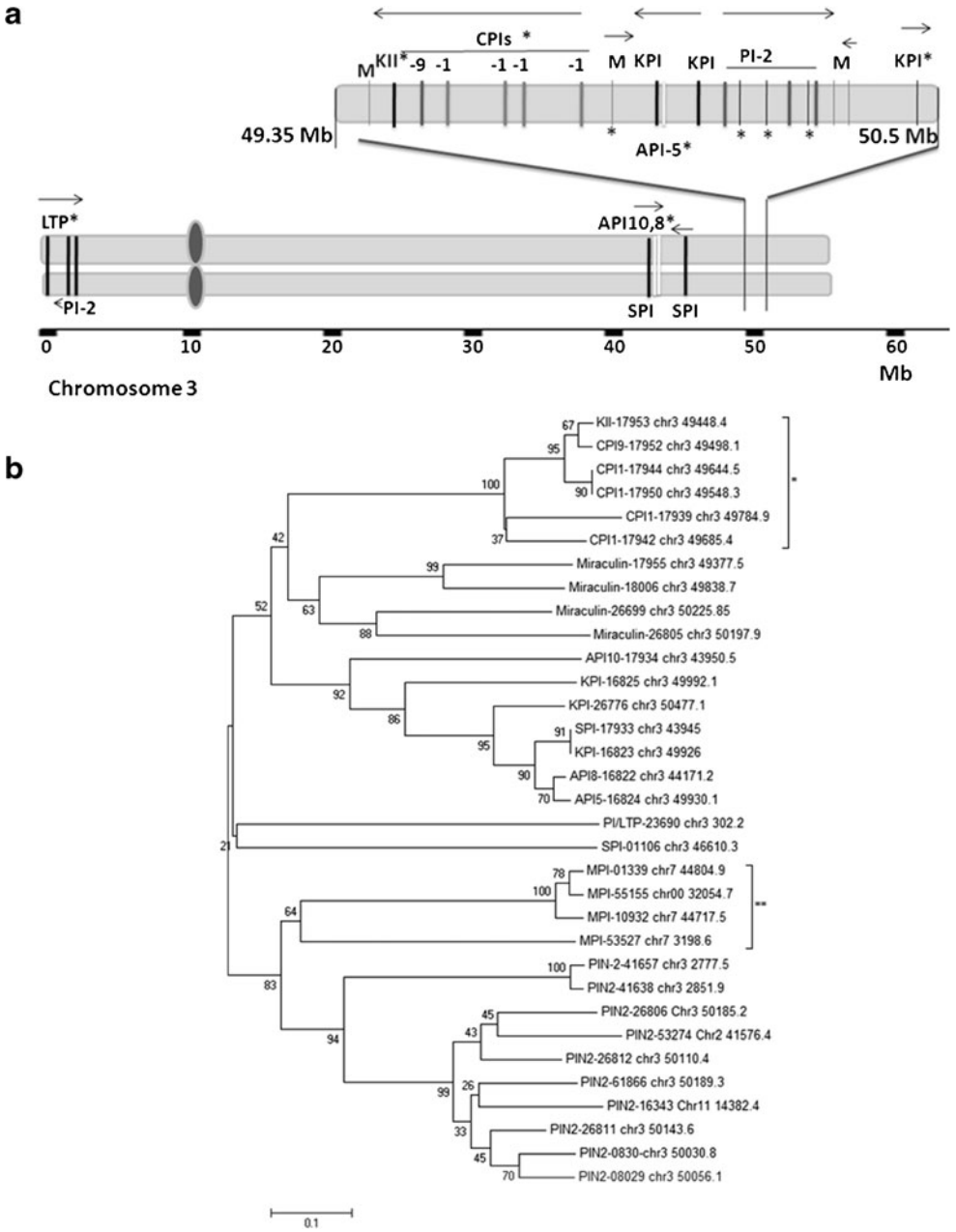


Fig. 4 Analysis of protease inhibitors in the *Solanum phureja* genome. **a** Schematic representation of protease inhibitor genes mapped in chromosome 3. Positions (kbp) are indicated. *Arrows* indicate gene orientation, *asterisk* indicates those clones that are present in the TIGR 10K array. API, aspartic protease inhibitors; CPI, cysteine protease inhibitors; KII, Kunitz-type tuber invertase inhibitor; KPI, Kunitz protease inhibitor; M, miraculin; MPI, metallocarboxyprotease inhibitors; PI-2, proteinase inhibitor type-2; SPI, serine protease inhibitor 7; TPL, protease inhibitor/seed storage/lipid transfer protein family protein. **b** Evolutionary relationships of 31 amino acid sequences from potato protease inhibitors. Included are the inhibitors shown in Fig. 5a plus metallocarboxypeptidase inhibitors (MPI) and two PI-2 located in chromosomes 2 and 11. In each case, chromosome position is indicated. The numbers beside the inhibitors correspond to the PGSC0003DMP accession was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 6.08613955 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar 2000) and are in the units of the number of amino acid differences per site. All positions containing gaps and missing data were eliminated. There were a total of 29 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013)

regulated. By qRT-PCR and Northern blot, we also detected that MPI expression correlates with the extension of the dark period, being highest in sprouts exposed for 20 days to continuous darkness confirming the microarray data and

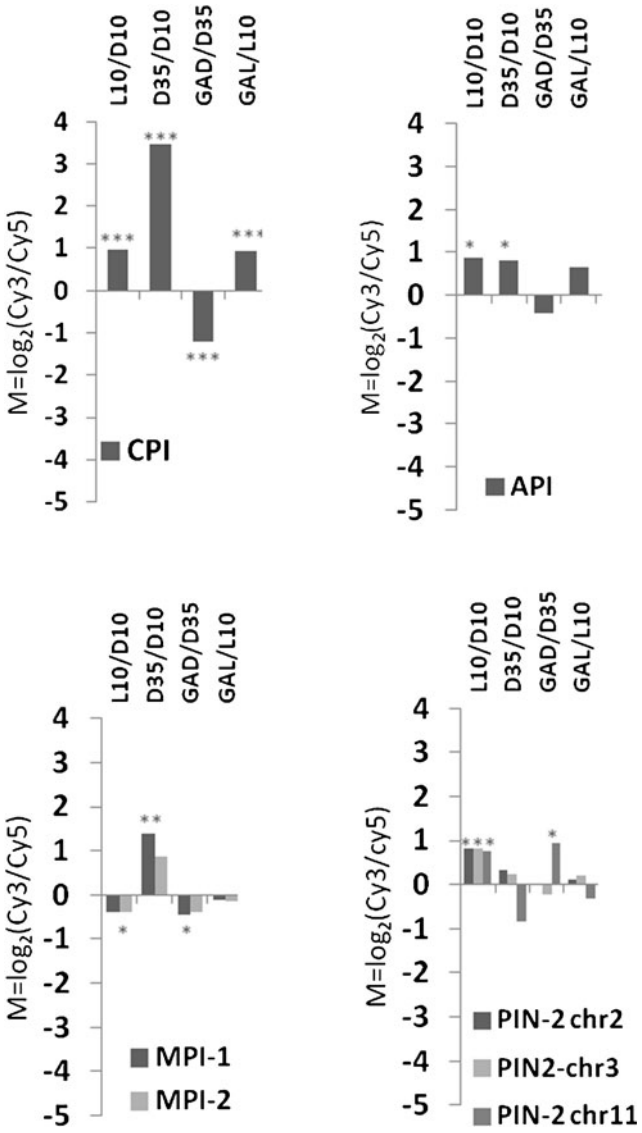


Fig. 5 Analysis of clones encoding different protease inhibitors in the hybridizations (L10 vs. D10; D35 vs. D10; DGA vs. D35; LGA vs. L10). **a** Cysteine protease inhibitors CPIs (clones STMCE08, STMCL26, STMCV60, STMCX33, and STMHQ67). **b** Aspartic PIs, API (clones STMCQ07, STMCQ55). **c** Metallocarboxypeptidase inhibitors MPIs (*1* STMIW76, *2* STMGL75). **d** PIN2 (STMIY57, STMCP16). Plotted are the M [$M = \log_2(\text{Cy3/Cy5})$] values. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.005$ (***)

suggesting that darkness has a direct effect on MPI expression. However, CPIs are induced once the dark period is extended. When GA was added to the media, it enhanced the expression of proteases and reduced the expression of inhibitors; this is similar to the effect of GA on germinating seeds where it induces the expression of proteases to digest storage proteins (Kiyosaki et al. 2007).

CPIs were shown to provide broad tolerance to drought, chilling, oxidation, or salt stress (Quain et al. 2014). We corroborated in silico that potato CPIs contain promoter sequences that possess the DNA elements ABRELATERD1 and MYCARTERD1 that were previously shown to be induced in dark conditions (Simpson et al. 2003). Comparison of sink and source tubers at both RNA and protein levels revealed that while patatin genes are almost exclusively expressed in sink tubers, the amount and number of patatin polypeptides are greatly reduced in the source situation. PMC accumulation in tubers precedes patatin deposition during the initial stages of tuber development (Weeda et al. 2010). In storage, the PMC content of tubers declines concomitant with a loss of patatin, and during the early stages of plant development, PMC levels decline precipitously, likely facilitating mobilization of nitrogen reserves to support the developing plants (Weeda et al. 2010). In accordance, transcriptome analysis of dormant and sprouted tubers using TIGR arrays reported that dormancy exit in tuber meristems is accompanied by decreased expression of a number of protease inhibitors including MPIs, CPIs, and APIs (Campbell et al. 2008). Thus, by modulating the activities of proteases, PMC and other inhibitors may play a role in regulating tuber protein content during development, storage, and sprouting. These studies were performed with dormant and sprouted tubers while our study compares sprouts in different developmental stages, and the up-regulation of protease inhibitors is observed after an extended dark period. It is possible that in D35 sprouts, CPIs are modulating other processes.

In planta cystatins inhibit the activity of cysteine proteases (CPs), enzymes that play key roles in physiological processes such as programmed cell death, senescence, defense, and storage protein mobilization (Martínez et al. 2012). The senescence-specific cysteine protease gene SAG12 has been shown to be induced (Parrott et al. 2007; Pourtau et al. 2006) or repressed (Noh and Amasino 1999) by sugars during senescence. The participation of CPs in intracellular protein catabolism for senescence and programmed cell death is of phytophysiological importance. Some CPs are induced in seeds for stress tolerance as in the case of drought and damage. Changes in selective synthesis or degradation of individual proteins could be important components in the coordinated response to carbon starvation (Graham 1996; Koch 1996). Brouquisse et al. (1998)) observed starvation-related proteolysis in sink tissues of 3-week-old maize (*Zea mays* L.) plants exposed to 48 h of darkness. As a consequence of this starving, plants no longer post resources to protein synthesis associated to photosynthesis, and metabolic efforts are re-directed. Autophagy is a conserved process that sequestrates and delivers macromolecules and organelles to the vacuoles or lysosomes for degradation. Autophagy in higher plants is involved in supplying nutrients during starvation and in promoting cell survival during senescence and during biotic and abiotic stresses. We provide novel information regarding the effect of extended darkness on sprouting, and we hypothesize that the prevention of CP activity is a sprout response to the prolonged absence of light, in the battle to subsist in hostile

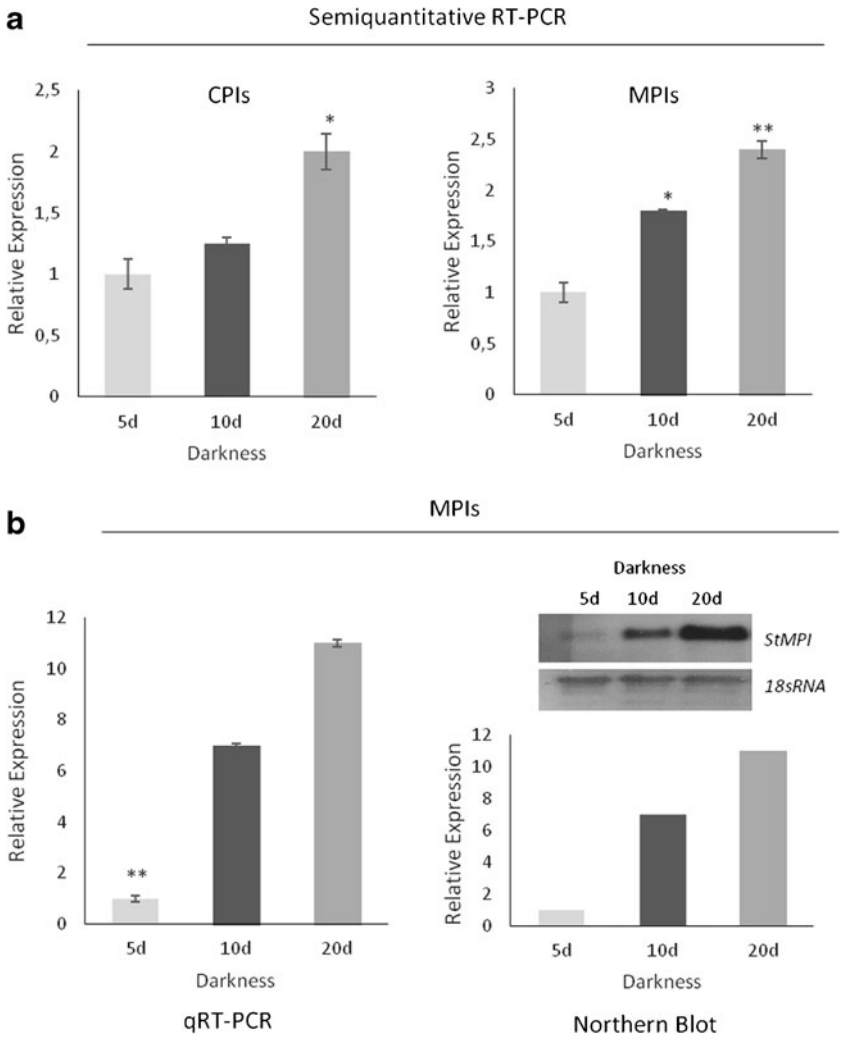


Fig. 6 Expression analysis of protease inhibitors genes in sprouts grown under continuous darkness for 5, 10, or 20 days. **a** Semi-quantitative RT-PCR of CPIs (*left*) and MPIs (*right*) transcripts. **b** Quantitative real-time PCR (*left*) and Northern blot analysis (*right*) of MPIs transcripts. Relative expression is plotted, quantification of PCR products was performed with ImageJ software and normalized using EFα1 transcript. *Error bars* represent standard deviation between technical replicates. RNA loading was checked by hybridization with a labeled 18s rRNA probe. *Asterisks* indicate significant differences. Data are from one of three independent experiments with similar results

conditions. Senescence is not triggered in young tissues and only upon very harsh conditions a young plant dies. Based on our findings, we propose that up-regulation of CPIs in the sprout inhibits CP activities triggered during dark-induced senescence and in PCD, in an attempt to survive and complete its life cycle.

Potato is the world's most important non-grain food crop, and successful storage of this crop is based on harvested tubers' ability to exhibit an endodormant state. Tubers tend to resume growth during storage, which is unsuitable for commercial purposes; it decreases the quality and affects the agriculture business. Recently, it was reported that overexpression of cereal cystatins in potato tubers delayed sprouting (Munger et al. 2015) suggesting the potential of endogenous CPs and CPIs as relevant targets for the development of potato varieties with longer storage capabilities. Understanding the role of the genes actively transcribed during the sprouting process is important to gain insights into the potato's life cycle proceeding and for future breeding improvements.

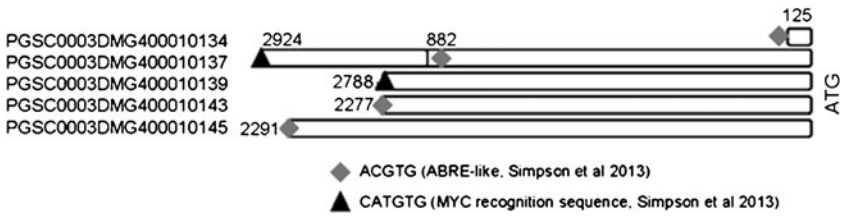


Fig. 7 The promoter regions of the genes corresponding to the protease inhibitors up-regulated in the array were analyzed in silico and dark-induced senescence associated motifs were detected. The motifs were described in Simpson et al. (2003). PGSC0003DMG400010134, cysteine protease inhibitor 1; PGSC0003DMG400010137, Kunitz-type invertase inhibitor; PGSC0003DMG400010139, cysteine protease inhibitor 1; PGSC0003DMG400010143, cysteine protease inhibitor 1; PGSC0003DMG400010145, cysteine protease inhibitor 9

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