Planta (2009) 230:73–84
DOI 10.1007/s00425-009-0928-0

ORIGINAL ARTICLE

Developmentally linked changes in proteases and protease inhibitors suggest a role for potato multicystatin in regulating protein content of potato tubers

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Received: 14 February 2009 / Accepted: 22 March 2009 / Published online: 5 April 2009

Abstract The soluble protein fraction of fully developed potato (*Solanum tuberosum* L.) tubers is dominated by patatin, a 40 kD storage glycoprotein, and protease inhibitors. Potato multicystatin (PMC) is a multidomain Cys-type protease inhibitor. PMC effectively inhibits degradation of patatin by tuber proteases in vitro. Herein we show that changes in PMC, patatin concentration, activities of various proteases, and their gene expression are temporally linked during tuber development, providing evidence that PMC has a role in regulating tuber protein content in vivo. PMC was barely detectable in non-tuberized stolons. PMC transcript levels increased progressively during tuberization, concomitant with a 40-fold increase in PMC concentration (protein basis) as tubers developed to 10 g fresh wt. Further increases in PMC were comparatively modest (3.7-fold) as tubers developed to full maturity (250 g). Protease activity declined precipitously as PMC levels increased during tuberization. Proteolytic activity was highest in non-tuberized stolons and fell substantially through the 10-g fresh wt stage. Cys-type proteases dominated the pre-tuberization and earliest stages of tuber development. Increases in patatin transcript levels during tuberization were accompanied by a notable lag in patatin accumulation. Patatin did not begin to accumulate substantially on a protein basis until tubers had reached the 10-g stage, wherein protease activity had been inhibited by approximately 60%. These results indicate that a threshold level of PMC (ca. 3 μg tuber^{-1}, 144 ng mg^{-1} protein) is needed to favor patatin accumulation. Collectively, these results are consistent with a role for PMC in facilitating the accumulation of proteins in developing tubers by inhibiting Cys-type proteases.

Keywords Potato multicystatin · Protein · Protease · Tuber development · *Solanum tuberosum* · Patatin · Tuberization

Abbreviations

Cys	Cysteine
DAP	Days after planting
EIA	Enzyme ImmunoAssay
FITC	Fluorescein isothiocyanate
LAH	Lipolytic acyl hydrolase
NPM	*p*-Nitrophenyl myristate
PCPI	Potato cysteine protease inhibitor
PI-1	Potato protease inhibitor I
PI-2	Potato protease inhibitor II
PKPI	Potato Kunitz-type protease inhibitor
PMC	Potato multicystatin
pNPP	*p*-Nitrophenyl phosphate
Ser	Serine

Introduction

Potato tubers are vegetative propagules that accumulate and store carbohydrate, lipid, and protein reserves during growth. These reserves will eventually be mobilized following a period of dormancy to support plant establishment. Protease activity increases in tubers during long-term storage (Kumar et al. 1999) and sprouting (Michaud et al. 1994), thus serving to mobilize protein nitrogen to support the growth of developing plants. Conversely, attenuation of

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proteolysis during tuber development is likely required to facilitate protein accumulation in tubers, and protease inhibitors may help regulate this process.

Proteases play key roles in a number of plant growth and developmental processes such as germination, cell differentiation, senescence, and programmed cell death (Palma et al. 2002; van der Hoorn 2008). Proteolysis is a complex process involving many different pathways and cellular compartments. Proteases in vivo are inhibited by endogenous inhibitors (Solomon et al. 1999; Rivard et al. 2007) or by compartmentation to prevent indiscriminant breakdown of proteins essential for metabolism (Wang et al. 2007). Protein degradation by proteases supplies amino acids for protein synthesis by degrading abnormal/misfolded proteins and for translocation to developing plants (Michaud et al. 1994) or seedlings (Sheokand et al. 2005) in the case of vegetative propagules or seeds, respectively.

Protease inhibitors comprise up to 50% of the soluble proteins in tubers, with the most abundant inhibitors, potato inhibitor II (PI-2) and the Kunitz protease inhibitor family, accounting for as much as 12 and 22% of the soluble protein fraction, respectively (Pouvreau et al. 2001, 2003). Potato protease inhibitors are classified according to their target proteases into seven families. These include potato inhibitor I (PI-1) and PI-2, potato cysteine protease inhibitor (PCPI), potato aspartate protease inhibitor (PAPI), potato Kunitz-type protease inhibitor (PKPI), potato carboxypeptidase inhibitor (PCI), and other Ser- and Cys-type inhibitors (Pouvreau et al. 2001). The low molecular weight Ser-type protease inhibitors (PI-1 and PI-2) also function as storage proteins. In addition to their roles in the wound-inducible defense response of potato leaves to insects (Green and Ryan 1972), some of these protease inhibitors may participate in regulating tuber protein content. Indeed, protease inhibitors have been shown to function in regulating protein deposition during chloroplast ontogeny (Prins et al. 2008) and in nitrogen remobilization during germination (Valdés-Rodríguez et al. 2007) and leaf senescence (Etienne et al. 2007).

Potato multicystatin (PMC) is a Cys-type protease inhibitor with eight inhibitory domains. PMC is distributed throughout the tuber as a soluble 85 kD monomer and as a crystalline tetramer concentrated beneath the periderm (Rodis and Hoff 1984; Walsh and Strickland 1993). PMC is susceptible to trypsin (Ser protease) digestion which yields 35-kD and 10-kD peptides, all with functional inhibitory domains (Walsh and Strickland 1993). While in vitro studies attribute insect anti-feedant activity to PMC (Orr et al. 1994), little is known about its intrinsic biological function(s) during tuber development, dormancy, and sprouting.

In vitro studies have demonstrated that PMC inhibits patatin degradation by tuber-derived Cys-proteases (Kumar et al. 1999). As a Cys-protease inhibitor, PMC may thus have a role in regulating protein accumulation in tubers during development by attenuating proteolysis and shifting the equilibrium between protein synthesis and catabolism toward synthesis. For this to occur, PMC synthesis, from gene expression to the accumulation of functional inhibitor in tubers, must precede or at least be concurrent with the accumulation of major storage proteins such as patatin early in tuber ontogeny. Detailed information linking such changes in PMC and other protease inhibitors to tuber development is lacking. This study focuses on defining changes in tuber protein content in relation to PMC levels and protease activity from tuber initiation to full maturity. We demonstrate that on a milligram protein basis, PMC increases more rapidly than patatin during tuber initiation, concomitant with precipitous decline in total- and Cys-protease activities. The data indicate that a threshold level of PMC is required in vivo (ca. 3 μg per tuber; 144 ng PMC mg^{-1} protein) to inhibit protease activity sufficiently (approximately 60%) to shift the equilibrium in patatin turnover toward accumulation.

Materials and methods

Plant material and tuber developmental stages

Tubers in different stages of development were obtained from field plots at the Washington State University Irrigated Agriculture Research and Extension Center at Othello, WA (46º 47.277′ N. Lat., 119º 2.680′ W. Long.). In preparation for planting, certified (G3, certified generation three from nuclear stock) cv. Ranger Russet seed-tubers were obtained directly from a local seed potato packing shed in early April each year of the study. Seed-tubers were hand cut into 50- to 64-g seedpieces and suberized at 10°C ± 0.5°C (95% RH) for 5 days prior to planting. Seedpieces were planted 20-cm deep in a Shano silt loam soil (classified as Andic Mollic Camborthid [Lenfesty 1967]) with a custom built two-row assist-feed planter. Four, 90-m long rows were planted 86 cm apart with seedpieces spaced 25 cm apart within each row. The plots were located under a linear move irrigation system. Soil moisture was maintained at a minimum of 65% of field capacity with the aid of soil moisture probes positioned throughout the field. Preplant and in-season fertilizer applications were based on soil tests and petiole analyses, respectively, following standard practices for long-season russet potatoes in the Columbia Basin. Herbicides, insecticides, and fungicides were applied as needed, according to standard practices.

Plants and tubers were hand harvested from the two center rows at approximately 10-day intervals from 62 to 176 days after planting (DAP). Four replicates of four consecutive plants per replicate were selected at random from

within the two center rows for each harvest. Foliar (above ground) fresh weight and tuber number and fresh weight were recorded. Foliar and tuber fresh weights were plotted versus days after planting (DAP) to establish the post tuberization developmental profile. Ten developmental stages (designated stages 9–18) defined post tuberization development.

Tubers were analyzed at each harvest (five tubers per replicate, four replicates) for protein, protease activity, PMC content, patatin, and associated gene expression, as described below. The tubers at each harvest were chosen to represent the average tuber fresh weight (g/tuber) at that point in the growing season. Twenty tubers were sampled at each harvest in four replicates of five tubers each. Tubers were halved longitudinally and the apical halves of 1.5-mm-thick central slices (periderm attached) from each of the five tubers were collectively frozen at –80°C and lyophilized. The dried tissue was weighed, ground (mortar and pestle), and sieved through a 60 mesh (0.246 mm) screen.

The earliest stages of tuber development were sampled during the period of tuberization from 40 to 54 DAP (see Knowles et al. 2008). Samples, ranging in development from non-tuberized stolons to 10-g tubers (Fig. 1), were collected from whole plants harvested with root systems intact. Tuberization stages I–IV were classified according to stolon and tuber morphology; stages V to VIII according to tuber weight (Fig. 1). Multiple samples of each of the eight stages were pooled from different plants to give four replicates of each stage (at least five tubers per replicate for stages V–VIII and 10–50 stolons/tubers per replicate for stages I–IV). The replicate samples of stages I–VIII were frozen intact in liquid N_2 and stored at –85°C until further analysis.

Protein extraction and analysis

Replicate samples of tuberization stages I–VIII were ground (mortar and pestle) to a fine powder in liquid N_2. Soluble protein was extracted from 1.5 g of the frozen powdered tissue, and from 300 mg lyophilized tuber tissue (post tuberization samples) with 3 mL potassium phosphate buffer (50 mM, pH 7.5) containing 0.5% (w/v) PVPP and 2 mM sodium bisulfite at 4°C. The slurry was vortexed and divided into equal portions. Protease inhibitors (Sigma) were immediately added to one portion of the extract to achieve final concentrations of 5.1 μM leupeptin, 4.1 μM chymostatin, 3.8 μM pepstatin, and 3.7 μM antipain. The extracts were incubated on ice for 20 min with occasional vortexing before centrifugation at 10,600*g* for 20 min at 4°C. Supernatants were stored at –20°C until analysis. The portion of extract supplemented with protease inhibitors was analyzed for soluble protein, PMC, patatin, and was also used for SDS–PAGE and characterization of proteases via activity gels. The portion of extract without exogenous protease inhibitors was used to assess total protease activity

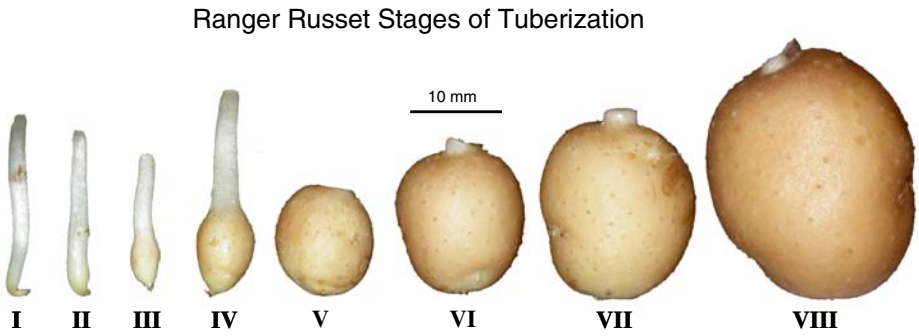

Fig. 1 Classification of tuberization and early stages (I–VIII) of development of cv. Ranger Russet tubers. Samples were collected from field-grown whole plants during the period of tuberization, 40–54 days after planting

Stage	Morphological and Weight Descriptions of Tuberization Stages
I	Hooked stolons with no swelling below the apical hook
II	Slight swelling below the apex begins to open apical hook
III	Further swelling in terminal 5 mm of stolon forces hook to open completely; developing tuber is less than twice the diameter of the stolon
IV	Longitudinal and radial expansion progress; developing tuber is approximately twice the diameter of the stolon.
V	0.6- to 1.5-g tuber
VI	1.5- to 2.5-g tuber
VII	2.5- to 5.0-g tuber
VIII	5- to 10-g tuber

(see below). Protein was quantified (Bradford 1976) using protease-free BSA (Sigma) as a standard.

SDS–PAGE and protease activity

Developmentally linked changes in tuber soluble proteins were characterized qualitatively by SDS–PAGE (10% gels, 20 μg protein per lane) (Laemmli 1970). The proteases in extracts of tuberization stages I–VI were separated on 10% gels containing 0.15% gelatin with or without 300 μg PMC. The PMC-containing gels were used to differentiate Cys-type proteases from other gelatinolytic proteases using a reverse zymography approach (see Oliver et al. 1997). Following electrophoresis (110 V, 120 min, 4°C), SDS was washed from the gelatin-containing gels by incubating (23°C) in 2.5% (w/v) Triton X-100 for 60 min. The gels were then incubated at 37°C overnight in 50 mM potassium citrate buffer (pH 6.5) containing 5 mM L-Cys, prior to staining with Coomassie blue. Destaining revealed the gelatinolytic protease activities, which appeared as achromatic bands on a dark blue background (Michaud et al. 1993, 1994; Kumar et al. 1999). Subsequent clearing of gelatin from the gels by incubating in trypsin (1900 BAEE units in 50 mM Tris, pH 7.5) revealed the presence of serine protease inhibitors, which remained as darkly stained bands due to inhibition of the trypsin-induced gelatinolytic activity.

Quantification of PMC

PMC was quantified by enzyme linked immunosorbent assay (ELISA) using purified PMC as a standard. To prepare the standard curve, PMC was first purified by sucrose density gradient centrifugation according to Rodis and Hoff (1984). The purified PMC was diluted to 0.132 ng protein μL^{-1} with 50 mM sodium carbonate buffer (pH 9.6). Diluted PMC was used to produce a standard curve ranging in concentration from 0 to 1.6 ng PMC protein per 500 μL. One hundred microliters of each standard were transferred (in triplicate) to the wells of a high binding, flat bottom, 96-well EIA (Enzyme ImmunoAssay) polystyrene plate (Corning Inc., Corning, NY) to produce a standard curve ranging from 0 to 0.32 ng PMC protein per well.

Protein extracts of stages I to VIII (tuberization stages) and 9 to 18 (post tuberization stages) were diluted 1:100 with 50 mM sodium carbonate buffer (pH 9.6). Samples (100 μL) of the extracts were then transferred to the EIA plate and incubated for 2 h at 37°C. Protein bound to the wells was blocked overnight at 4°C in blocking buffer (20 mM Tris, 150 mM sodium chloride, 0.05% (w/v) Tween 20, pH 7.6). The blocking buffer was removed and 200 μL of polyclonal anti-PMC (1:48,000) were added to each well, followed by 2 h incubation at room temperature. The wells were rinsed three times with blocking buffer, secondary antibody (anti-rabbit, 1:1000) was added to each well, and the plates were incubated for 2 h at room temperature. The samples were then rinsed (as above) with blocking buffer, 200 μL of alkaline phosphatase yellow (pNPP, Sigma) were added to each well, and the plates were incubated for 2 h (32°C) prior to measuring A_{405}.

Protease activity

Protease activity was assayed in stolon/tuber extracts prepared without protease inhibitors using FITC-casein as substrate (Sigma) (Kumar et al. 1999). The assay was initiated by adding 300 μL of sample extract to 200 μL of potassium phosphate buffer (50 mM, pH 6.5) containing 3.7 mM DTT and 500 μg FITC-casein. The reaction was incubated at 37°C in the dark for 90 min and terminated by adding 100 μL 50% (w/v) TCA to each sample. Protein and unreacted FITC-casein were pelleted (1,610g, 22 min, 4°C) and 350 μL of the supernatant were transferred to 1 mL Tris buffer (1 M, pH 8). The sample was vortexed and triplicate aliquots of 300 μL were added to the wells of a 96-well white polystyrene flat-bottomed medium-binding micro titre plate (Corning Inc., Corning, NY). Emission (520 nm, excitation 500 nm) was measured on a fluorescence spectrophotometer (Varian Cary Eclipse, Varian Inc., Palo Alto, CA). Protease activity was expressed as relative proteolytic units (emission 520 nm) per mg protein.

Quantification of patatin

Levels of patatin (40 kD), the major lipolytic acyl hydrolase (LAH) in potato (Andrews et al. 1988; Macrae et al. 1998), were estimated as LAH activity using *p*-nitrophenyl myristate (NPM) as substrate (Lulai et al. 1986; Lojkowska and Holubowska, 1989; Zabrouskov et al. 2002). NPM (20 mg) was solubilized in 6 mL acetone containing 1.2 mL Triton X-100. The acetone was evaporated under N_2 and the remaining solution dissolved in 34.5 mL of potassium phosphate buffer (0.1 M, pH 7.5). The reaction contained 300 μL of substrate, 1.7 mL potassium phosphate buffer (as above), and 25 μL of extract. The release of nitrophenol was monitored continuously for 1 min at A_{400} on a Varian Cary double beam spectrophotometer (Varian Inc., Palo Alto, CA). LAH activity was calculated using the extinction coefficient of 17.42 mM^{-1} cm^{-1} (Lulai et al. 1986) and expressed as nmol nitrophenol released min^{-1} mg^{-1} protein. Patatin levels were also estimated by densitometry scanning of SDS–PAGE gels.

RNA extraction and RT-PCR

RNA was extracted from frozen or lyophilized tissue using a modified method (Kumar et al. 2007). Following DNase

treatment (TURBO DNA-*free*™ kit, Ambion, Foster City, CA), first-strand cDNA was synthesized from 1 μg RNA (ThermoScript™ RT-PCR kit, Invitrogen, Carlsbad, CA). PCR was carried out with GoTaq® Green DNA polymerase as per the manufacturer's instructions (Promega Corporation, Madison, WI). Primers for PMC, patatin, PI-1, PI-2, PKPI, and tubulin were obtained from Invitrogen (Carlsbad, CA). The forward and reverse primer sequences, gene accession numbers, and amplified PCR product lengths are provided in Table 1.

Data analysis and presentation

Growth and development data were subjected to analysis of variance with time (days after planting) and foliar or tuber fresh weight as independent and dependent variables, respectively. Sums of squares were partitioned into polynomial (linear, quadratic, cubic) trends and the data plotted accordingly. Similarly, tuber protein, patatin and PMC concentrations, and protease activity were regressed over time or versus PMC level. Coefficients of determination are reported along with significance levels (*P*-values) for the correlation coefficients. Data are plotted with 95% confidence intervals.

Results

Characterization of tuber development

Field plots of cv. Ranger Russet were established to provide 18 stages of tuber development over a 176-d growing season. The crop was grown according to commercial practices for long-season russet cultivars in the Columbia Basin. The study was repeated over three seasons (2005, 06, 07) with similar results and data from the 2006 season are presented as representative. Eight stages (I–VIII) of development (Fig. 1) during tuberization (40–54 days after planting) were combined with samples taken during the post tuberization bulking phase to represent the complete developmental profile (Fig. 2). Typical for long-season russet cultivars in the Columbia Basin (Knowles et al. 2008), the trend in foliar fresh weight was cubic ($Y = -13.5 + 0.391X - 2.55e^{-3}X^2 + 4.18e^{-6}X^3$, $R^2 = 0.98$, $P < 0.001$) with a maximum at 100 days after planting (DAP). While foliar biomass declined from 110 DAP to season end, bulking continued until 150 DAP, indicating that photosynthesis was sufficient to support tuber growth well beyond the onset of foliar decline. Proteases are involved in regulating the process of leaf senescence (Thoenen et al. 2007 and references therein) and determining the role of protease inhibitors in this process warrants further investigation.

The harvest index (tuber fresh weight divided by foliar plus tuber fresh weight) at foliar maximum was 53% (Fig. 2). The post tuberization stages of development (stages 9–18, Fig. 2) spanned the tuber bulking phase from 62 to 176 DAP, where tuber fresh weight increased from 25 to 250 g. Tuber growth during this period was sigmoidal and best described by a cubic polynomial ($Y = -21.5 - 2.22X + 0.056X^2 + 1.97e^{-4}X^3$, $R^2 = 0.98$, $P < 0.001$). Samples of each stage of tuber development were analyzed for relative changes in protein, PMC, and protease activity.

Developmental changes in tuber protein, patatin, protease activity, and PMC

Protein concentration declined 32% on a tuber dry matter basis (from 37 to 25 mg g^{-1} dry wt) as tubers developed from tuberization stage I to full maturity (stage 18) (mg

Table 1 Forward (F) and reverse (R) primer sequences for the amplification of transcripts of potato patatin, potato multicystatin (PMC), potato protease inhibitors 1 and 2 (PI-1, PI-2), and potato Kunitz-type protease inhibitor (PKPI). RT-PCR results are shown in Fig. 7 (TUBST 1, tubulin)

Gene	Primer sequence (5′ → 3′)	Product	Accession	Reference
TUBST1	**F**-AAATGTGCAGAACAAGAACTCATCC	420 bp	Z33382	Taylor et al. (1994)
	R-CATAACAAGTTCACTTTGGCAG			
Patatin	**F**-GTTATACAGCAAATGACTGAGGCAGC	250 bp	DQ274362	Stupar et al. (2006)
	R-CCTCTTTAGAGCTTCCTCATAGGTTTC			
PMC	**F**-CAATCGTAGGAGGCCTTGTCGAT	350 bp	L16450	Waldron et al. (1993)
	R-CGAGCAAGTTCTTGAAACTTGGTGT			
PI-1	**F**-CTCACATCATTGTTTTCTTTCTTCTTGCAAC	240 bp	AY496262	Van Den Broek et al. (2004)
	R-CTAAAATCCTTTGTGACTGGAGAACCATTC			
PI-2	**F**-GCCCACGTTCAGAAGGAAGTCCG	320 bp	X03778	Sanchez-Serrano et al. (1986)
	R-GCAGGGTACATATTTGCCTTGGGCTC			
PKPI	**F**-GTACTCCCTGAAGTTTATGACCAAGATGG	355 bp	X56874	Yamagishi et al. (1991)
	R-AGATGTCGTTTTCATTTCCTACGTTACCAC			

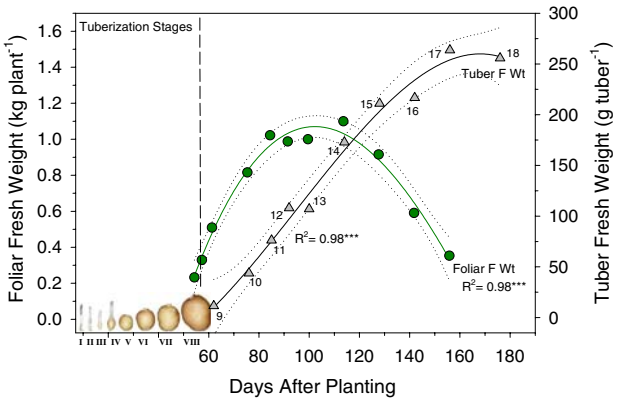

Fig. 2 Foliar and tuber growth of cv. Ranger Russet under late-season management at Othello, WA during 2006. Planting date was April 11 and vines were removed 156 days after planting. Plants were harvested at approximately 10-day intervals over the 176-day growing season to provide tuber samples for protein, protease activity, and protease inhibitor analyses. Stages I–VIII were collected during the tuberization phase of development from 40 to 54 days after planting. *Dotted lines* represent 95% confidence intervals

protein g^{-1} dry wt = 42.2 – 0.219DAP + 7.14e^{-4}DAP^2, $R^2 = 0.68$, $P < 0.001$). On a whole tuber basis, soluble protein levels increased 230-fold during the early stages of tuber development (stages I–VIII) but less so (29-fold) during the bulking phase from stages 9–18 (Fig. 3a). Protease activity was highest in non-tuberized stage-I stolons and fell precipitously to 19% of initial activity as stolons began to swell at tuber induction (stages II and III). Hence, the substantial increase in protein deposition during tuberization was accompanied by considerable decline (85%) in protease activity in the soluble protein fraction, most of which occurred during development from stage I to V. Protease activity continued to decline during tuber bulking to stage 13 (100 DAP) and then remained constant at levels approaching the limits of detection as tubers developed to full maturity.

Changes in the patatin (LAH activity) content of tubers on a whole tuber basis during the tuberization and post tuberization stages of development mirrored those described above for soluble protein (Fig. 3b). As a fraction of total soluble protein, patatin increased from less than 0.1% to only 7.5% of that in fully developed (stage 18) tubers during tuberization from stages I to VIII. Hence, the greatest increases in patatin on a milligram protein basis occurred as tubers bulked from stage 9 to 18 (62-176 DAP). Patatin's contribution to total soluble protein increased from less than 5–15% as tubers developed from stage I to VIII and 15–40% from stage 9 to 18 (Fig. 4). Note that the greatest increases in patatin during the bulking phase of tuber development occurred when protease activity had decreased by 90% (protein basis) (Fig. 3a).

In contrast to patatin, the greatest increases in PMC, on both a whole tuber and milligram protein basis, occurred

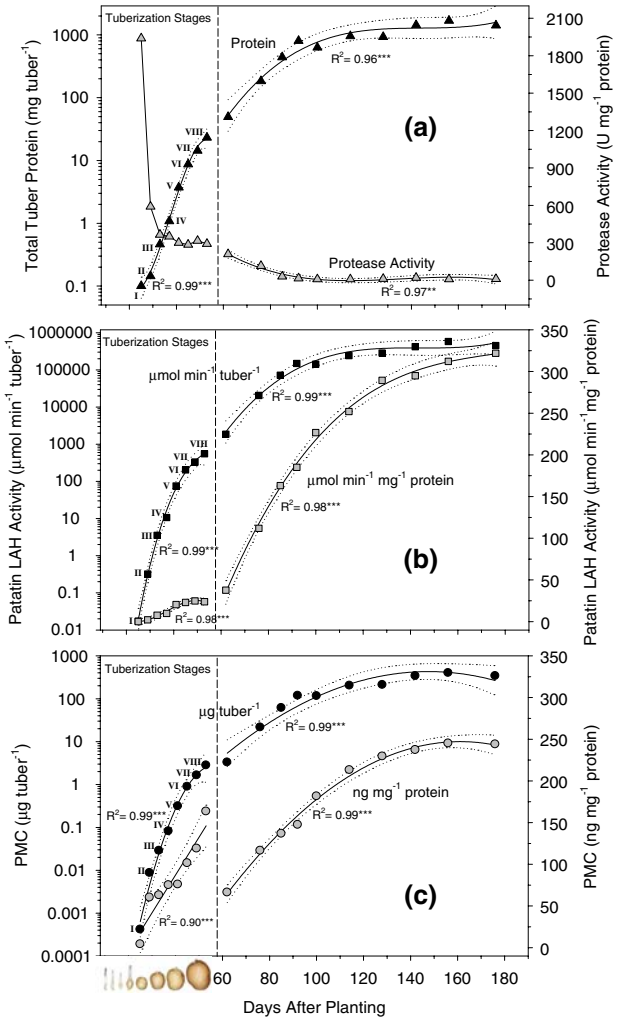

Fig. 3 Changes in tuber soluble protein and total protease activity (**a**), patatin (**b**), and PMC (**c**) with days after planting on a total tuber (*black symbol*) and protein (*gray symbol*) basis. The developmental stages corresponding to each point are defined in Figs. 1, 2. Protease activity (**a**, *gray triangles*) was assessed spectrofluorometrically using FITC-casein as substrate. Soluble protein (*black triangles*) was quantified using the Bradford method with BSA as a standard. Patatin levels (**b**) were assessed as lipolytic acylhydrolase activity using *p*-nitrophenyl myristate as substrate. PMC was quantified with ELISA using purified PMC as standard. The *vertical dashed lines* indicate different tissue sampling techniques for the tuberization versus bulking stages of tuber development. For stages I–VIII, whole stolons/tubers (i.e. intact organs) were analyzed. For stages 9–18, complete longitudinal slices of tuber tissue were analyzed to represent the whole tuber. *Dotted lines* represent 95% confidence intervals

during tuberization (stages I–VIII) (Fig. 3c). PMC increased from 4.1 to 163 ng mg^{-1} protein as tubers developed from stolons (stage I) to stage VIII tubers, a 40-fold increase. PMC continued to increase in tubers through the bulking phase; reaching a maximum of 247 ng mg^{-1} protein in 225 to 250-g tubers (stages 16–18, 142–176 DAP). On a whole tuber basis, patatin LAH activity was highly correlated with PMC content. Patatin LAH activity

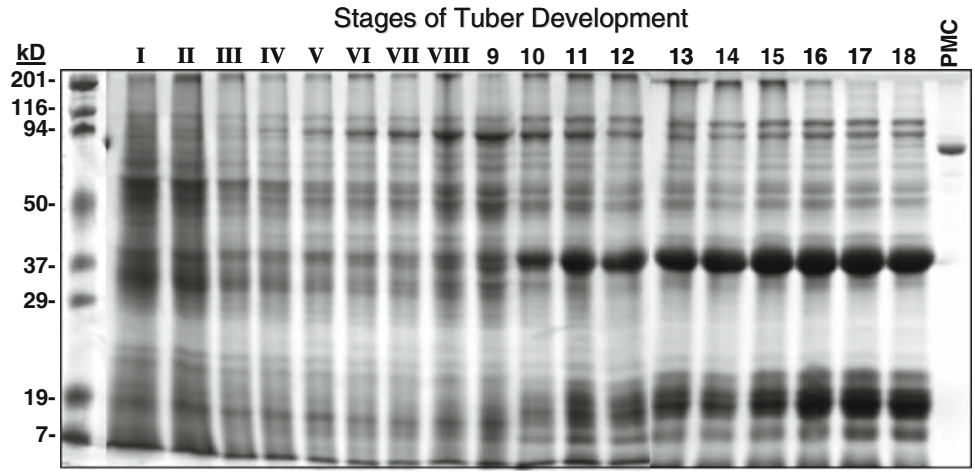

Fig. 4 SDS–PAGE of soluble proteins (20 µg per lane) from different stages of tuber development. The developmental stages corresponding to each lane are defined in Figs. 1, 2. Purified PMC (85kD, 5 µg) is shown in lane 19. (Patatin ca. 40 kD; PI-1 and 2 ca. 10 kD; PKPI ca. 20 kD)

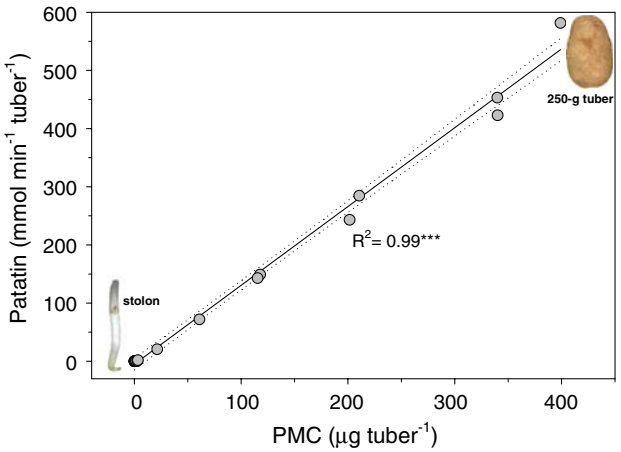

Fig. 5 Correlative relationship between patatin and PMC levels (whole tuber basis) during 18 stages of tuber development from non-tuberized stolons to fully mature, 250-g tubers. Note that the data points representing the earliest stages (I–VIII) cannot be resolved because of the scale. *Dotted lines* represent 95% confidence intervals. ***$P < 0.001$ for correlation coefficient

increased by 1.35 µmol min^{-1} for every microgram increase in PMC content of tubers ($R^2 = 0.99$, $P < 0.001$) (Fig. 5).

Visualization of proteins and proteases

Changes in protein profiles with development were assessed by SDS-PAGE (Fig. 4). Patatin appeared as a distinct band (ca. 40 kD) as early as stage III and gradually increased through stage 9. The greatest increase in patatin was evident during development from stage 9 (11 g fresh wt, 62 DAP) to 15 (210 g fresh wt, 128 DAP), after which levels remained relatively constant to full maturity (stage 18, 176 DAP). Note that the changes in patatin levels with tuber development as analyzed by SDS–PAGE were consistent with those for patatin LAH activity (Fig. 3b).

The Ser-type protease inhibitors, PI-1, PI-2, and PKPI, were barely visible as approximately 10 kD and 20 kD bands (Fig. 4) during early tuber development and remained at relatively low levels until stage 10 (44 g fresh wt, 76 DAP). These inhibitors then increased progressively and substantially in concentration as tubers developed from stage 10 to 17 (44–260 g, 76-156 DAP). These results were further confirmed by visualization of trypsin protease inhibitors (PI-1 and 2) (see Discussion).

Changes in proteolytic activity profiles were also compared. Proteases were separated by SDS-PAGE and assessed as gelatinolytic activity in the presence or absence of PMC. Consistent with trends in total protease activity (FITC-casein hydrolysis, Fig. 3a), gelatinolytic activity was highest during stages I–III and decreased substantially with further development (Fig. 6). At least five protease isoforms were observed as tubers developed from stolons to stage III. Further development of tubers to stage IV was accompanied by loss of the three lowest molecular weight isoforms and a significant reduction in activity of the two remaining isoforms. Gelatinolytic protease activity continued to decline as tubers developed through stage VI, beyond which activity was non-detectable with this method. Incorporation of PMC into the gel matrix completely inhibited gelatinolytic activity regardless of developmental stage, indicating that Cys-type proteases dominated the early stages of tuber development.

Changes in patatin and protease inhibitor transcripts

Relative changes in the transcript levels of patatin, PMC, PI-1, PI-2, and PKPI were compared as tubers developed from stage I to 18 (Fig. 7). Patatin gene expression was barely detectable in non-tuberized stolons but increased progressively from tuber initiation (stage II) through stage VI (1.5- to 2.5-g tubers). PMC gene expression was also detected at the onset of tuberization; however, maximum

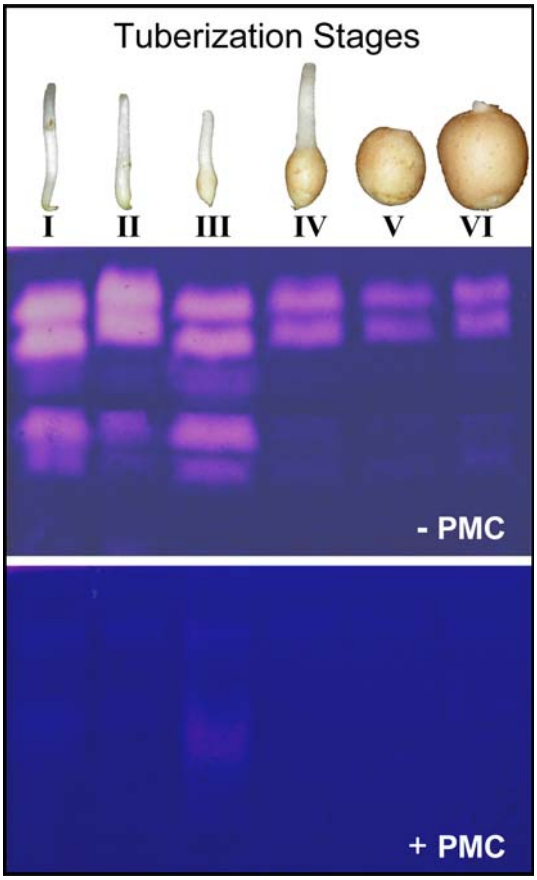

Fig. 6 Visualization of protease activity in tubers during tuberization from stages I to stage VI. Proteins (25 µg/lane) were separated by SDS–PAGE on gelatin-containing gels in the absence (*top*) or presence (*bottom*) of PMC (300 µg). Following electrophoresis and incubation of the gels, protease (gelatinolytic) activity appeared as achromatic bands when stained with Coomassie blue. Note that PMC completely inhibited gelatinolytic activity, indicating that Cys-type proteases dominate the early stages of tuber development

expression was not apparent until stages VIII to 10, lagging that of patatin. Expression of the Ser protease inhibitors PI-1, PI-2, and PKPI were also examined. PI-1 and -2 were constitutively expressed throughout all stages of tuber development. PKPI transcript was barely detectable in non-tuberized stolons, increased to a maximum as tubers developed through stage IV, and remained at a constant and high level as tubers developed further to full maturity.

Discussion

Protease inhibitors have been implicated in plant defense for their ability to inhibit exogenous proteases (Ryan 1990; Siqueira-Júnior et al. 2002; Goulet et al. 2008) and interfere with digestion of protein in the midgut of insects (Orr et al. 1994; Lecardonnel et al. 1999; Brunelle et al. 2005). However, there is increasing evidence that protease inhibitors also participate in the regulation of endogenous proteases (Rivard et al. 2007; Prins et al. 2008). Cys-proteases are involved in deposition and degradation of storage proteins, senescence, programmed cell death, and stress signaling (Grudkowska and Zagdańska 2004; van der Hoorn 2008). These processes must be tightly regulated to prevent unabated protease-mediated catabolism of proteins. Evidence suggests that protease inhibitors are involved in the fine control of proteolysis thereby affecting various aspects of plant development (Sin et al. 2006).

Potato multicystatin inhibits tuber- (Kumar et al. 1999) and leaf-derived proteases in vitro (Popovič and Brzin 2007). PMC exists as insoluble tetrameric crystals (340 kD) and/or in soluble monomeric form in potato tubers (Rodis and Hoff 1984). PMC is inactive in its crystalline form, which is likely a mechanism by which cells can store

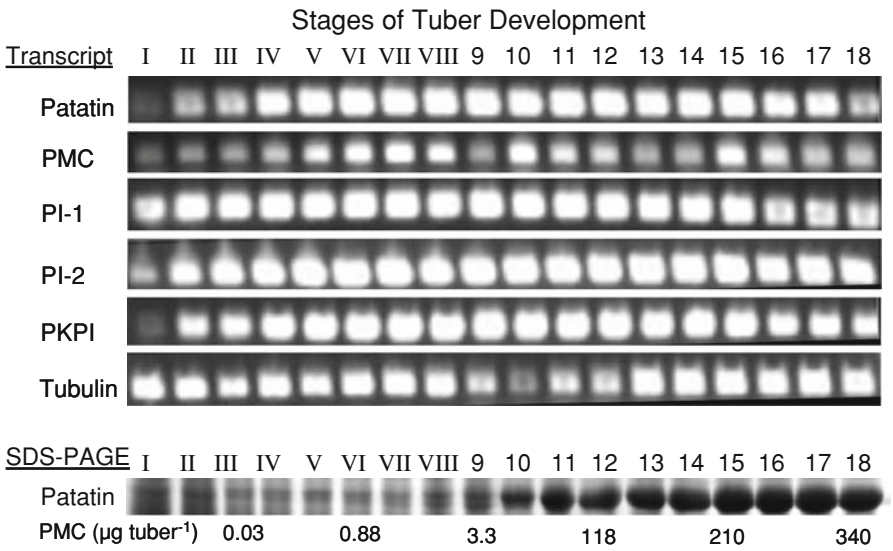

Fig. 7 RT-PCR analysis of patatin, PMC, and Ser protease inhibitor (PI-1, PI-2, PKPI) transcript levels in developing tubers. The developmental stages corresponding to each lane are defined in Figs. 1, 2. SDS–PAGE analysis of changes in patatin (20 µg/lane) levels during development (*bottom*) is provided for comparison with transcript levels. Note the lag in patatin accumulation despite high transcript levels early in development. PMC levels at select stages of tuber development are also indicated

massive amounts of Cys-protease inhibitor for protection from insects and control of endogenous proteases (Nissen et al. 2009). Transition of PMC to the soluble monomeric (ca. 85kD) form yields active inhibitor and is dependent on pH and inorganic phosphate concentration (Nissen et al. 2009). Hence, control of both PMC synthesis and structure are mechanisms by which protease activity can be modulated to affect key aspects of metabolism and tuber development.

The temporal relationships among PMC deposition, decreasing protease activity, increasing patatin levels, Ser protease inhibitor levels, and their gene expression provide correlative evidence that PMC is involved in regulating protein content during tuber development. While synthesis of PMC occurs throughout tuber development, it precedes the accumulation of patatin and Ser protease inhibitors during tuberization. Protease activity (esp. Cys-type) in non-tuberizing stolons is high and must be inhibited (Fig. 6) to facilitate the accumulation of storage and other proteins in developing tubers. The temporal relationships indicate that PMC is expressed, begins to increase in tubers early in development, and may need to reach a threshold level to inhibit proteases sufficiently to facilitate patatin accumulation. As PMC levels increased exponentially to about 3 µg per tuber through the 10-g fresh weight stage (stage VIII), protease activity declined linearly (Fig. 8). Despite early expression, patatin did not accumulate appreciably until stage VIII, and then increased substantially as PMC levels increased from 3 to 395 µg per tuber. Furthermore, Ser protease inhibitor proteins were non-detectable until stage 9 (Figs. 4, 9), despite constitutive expression at the onset of tuberization (Fig. 7). Hence, the increase in patatin and Ser protease inhibitors (Fig. 9) began only after protease activity was inhibited by

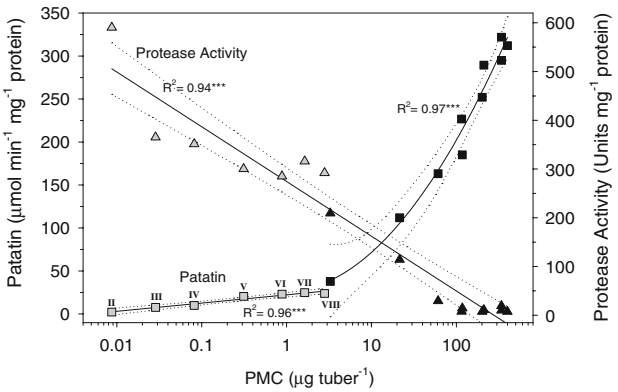

Fig. 8 Changes in patatin and protease activity with increasing levels of PMC in tubers during development from stage II to 18. The developmental stages are defined in Figs. 1, 2. Stages II–VIII (*gray symbols*), whole stolons/tubers (i.e. intact organs) were analyzed. For stages 9–18 (*black symbols*), complete longitudinal slices of tuber tissue were analyzed to represent the whole tuber. Note that changes in tuber PMC levels are exponential. *Dotted lines* represent 95% confidence intervals. ***$P < 0.001$ for correlation coefficients

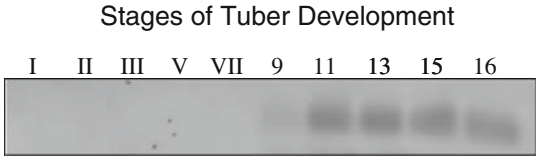

Fig. 9 Visualization of trypsin protease inhibitors (ca. 10 kD) in tubers of selected developmental stages (defined in Figs. 1, 2). Proteins (35 µg/lane) were separated by SDS–PAGE on gelatin-containing gels. Following electrophoresis the gels were incubated with trypsin to clear gelatin from the gels. The protease inhibitors attenuated proteolysis of the gelatin by trypsin, thus appearing as *darkly* stained bands (stages 9–16)

approximately 60% (Fig. 8), suggesting that a threshold level of PMC (ca. 3 µg tuber^{-1}, 144 ng mg^{-1} protein) is needed to favor patatin accumulation.

Figure 10 depicts a possible mechanism by which proteases and protease inhibitors may interact to shift the equilibrium from protein catabolism to accumulation in developing potato tubers. In the earliest stages of tuber development, protease activity is high and likely prevents protein accumulation at a rate corresponding to (predicted from) the high gene transcript levels. The presence of Cys-type proteases in non-tuberized stolons would prevent the accumulation of patatin and Ser protease inhibitors. Tuber-derived Cys-proteases degrade patatin (Kumar et al. 1999) and may degrade Ser protease inhibitors (Huang et al. 2005). Once PMC levels attain the threshold necessary to inhibit Cys-protease activity sufficiently (ca. 60%), patatin turnover would shift to favor accumulation (Fig. 8). The developmentally-linked expression and biochemical data reported herein support these interrelationships. However, further research is needed to unequivocally define the interplay among the various types of protease inhibitors and their target proteases during tuberization.

PMC may also prevent premature proteolysis of storage proteins in fully developed tubers. As vegetative propagules, tubers accumulate all the components necessary to support new growth during plant establishment. These components include protease inhibitors, storage proteins, and the proteases needed to initiate hydrolysis of storage proteins (Dunaevsky and Belozersky 1989; Tiedmann et al. 2001). Proteases are inactive and stabilized in the presence of their inhibitors (Yamada et al. 2000, 2001). Thus, the disappearance or inactivation of protease inhibitors at the onset of germination or sprouting may be necessary for protease-mediated degradation of storage proteins for nitrogen mobilization. Indeed, PMC levels decline during sprouting of potato seed-tubers, concomitant with an increase in protease activity and a decrease in patatin and other soluble proteins (Weeda et al. 2009), indicating a role for PMC in regulating mobilization.

Modulating protease inhibitor levels through molecular techniques (RNAi, sense/antisense, etc.) should provide unequivocal evidence of their role in regulating the protein

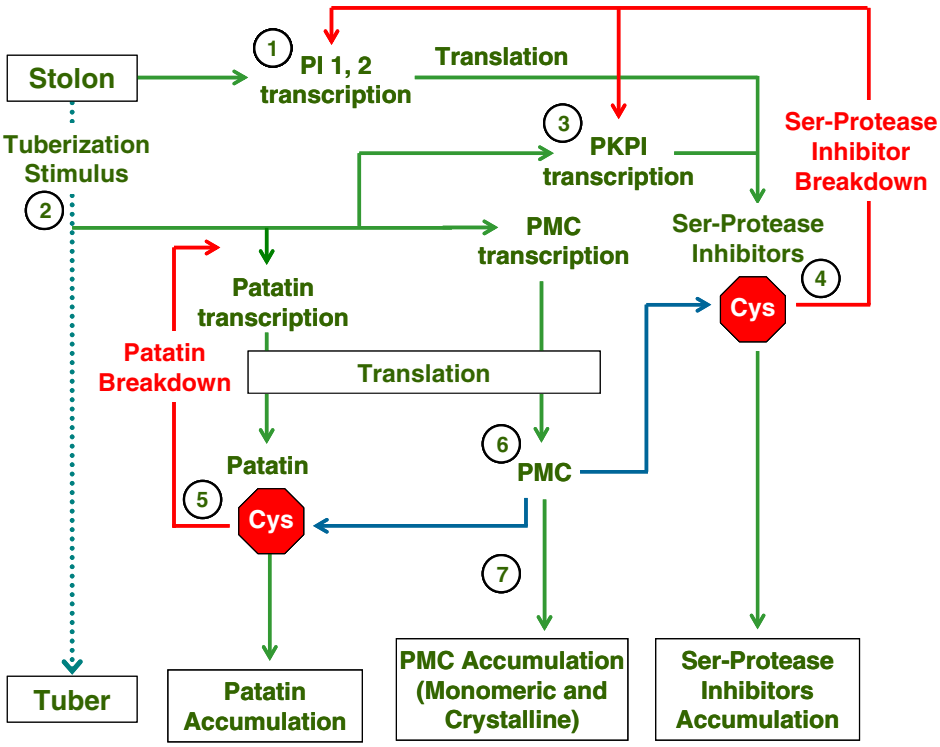

Fig. 10 Schematic illustrating a possible mechanism of PMC-mediated regulation of protein deposition during tuber development. PI-1 and PI-2 gene expression occurs in non-tuberized stolons (1) and is constitutive throughout tuber development (Fig. 7). When the tuberization stimulus (2) induces swelling of the stolon tips, PMC, patatin, and PKPI gene expression is initiated. PKPI transcript levels soon reach maximum intensity and remain constitutive throughout tuber development (3). However, the protein levels of the Ser protease inhibitors do not reflect the observed transcript levels and do not appear until after stage 9 (Figs. 4, 9). Ser protease inhibitors are susceptible to Cys-proteases (Huang et al. 2005); thus, the accumulation of Ser protease inhibitor proteins may be delayed (4) by proteolysis (Cys). Patatin transcript was barely detectable in stolons (stage I), increased to a maximum in 1.5- to 2.5-g tubers (stage VI), then maintained high levels throughout development. However, patatin protein accumulation rates do not reflect observed high transcript levels which may be due to the susceptibility of patatin to Cys-proteases (Kumar et al. 1999). Cys-proteases dominate early stages of tuber development (Fig. 6) and we speculate that the Cys-proteases (Cys) hydrolyze patatin protein, thus delaying its accumulation on a protein basis (5). PMC gene expression is delayed relative to patatin gene expression, but PMC protein accumulation precedes that of patatin on a protein basis (Fig 3). As a Cys-protease inhibitor, PMC may need to reach a threshold level (ca 3 µg tuber^{-1}, 144 ng mg^{-1} protein) to attenuate Cys-proteases sufficiently to shift patatin and Ser protease inhibitor turnover to favor accumulation (6). PMC initially accumulates in its monomeric form (7) but as its concentration increases, the crystalline tetramer is formed (Nissen et al. 2009). (Cys, cysteine protease)

content of developing tubers, and may provide strategies for enhancing tuber protein content and the nutritional value of potato through breeding and/or transgenic approaches. Determining the enzymatic or nonezymatic mechanism(s) regulating PMC content and activity as a protease inhibitor will provide further insight into the role of proteases and protease inhibitors in tuber ontogeny.

Acknowledgments Financial support provided by grants from the USDA/ARS, USDA/CSREES, Washington State Potato Commission and WSU Agricultural Research Center is gratefully acknowledged.

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