

Tubers from potato lines expressing a tomato Kunitz protease inhibitor are substantially equivalent to parental and transgenic controls

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Summary

Recombinant protease inhibitors represent useful tools for the development of insect-resistant transgenic crops, but questions have been raised in recent years about the impact of these proteins on endogenous proteases and chemical composition of derived food products. In this study, we performed a detailed compositional analysis of tubers from potato lines expressing the broad-spectrum inhibitor of Ser and Asp proteases, tomato cathepsin D inhibitor (*SCDI*), to detect possible unintended effects on tuber composition. A compositional analysis of key nutrients and toxic chemicals was carried out with tubers of *SCDI*-expressing and control (comparator) lines, followed by a two-dimensional gel electrophoresis (2-DE) proteomic profiling of total and allergenic proteins to detect eventual effects at the proteome level. No significant differences were observed among control and *SCDI*-expressing lines for most chemicals assayed, in line with the very low abundance of *SCDI* in tubers. Likewise, proteins detected after 2-DE showed no quantitative variation among the lines, except for a few proteins in some control and test lines, independent of *slcDI* transgene expression. Components of the patatin storage protein complex and Kunitz protease inhibitors immunodetected after 2-DE showed unaltered deposition patterns in *SCDI*-expressing lines, clearly suggesting a null impact of *slcDI* on the intrinsic allergenic potential of potato tubers. These data suggest, overall, a null impact of *slcDI* expression on tuber composition and substantial equivalence between comparator and *SCDI*-expressing tubers despite reported effects on leaf protein catabolism. They also illustrate the usefulness of proteomics as a tool to assess the authenticity of foods derived from novel-generation transgenic plants.

Keywords: compositional analysis, Kunitz protease inhibitors, protein allergens, proteomics, substantial equivalence, tomato cathepsin D inhibitor, transgenic potato tubers.

Introduction

Numerous studies reported the potential of recombinant protease inhibitors as useful antinutritive compounds to protect crop plants from herbivory or pathogenic infection (Michaud, 2000; Haq *et al.*, 2004). Ser protease inhibitors, in particular, have been readily identified as potential candidates for the production of insect-resistant transgenic crops (Hilder *et al.*, 1987; Johnson *et al.*, 1989; Duan *et al.*, 1996; Xu *et al.*, 1996), and their usefulness to

reduce insecticide loads in the field has been documented in recent years (Huang *et al.*, 2005; Qiu, 2008). Most protease inhibitors in plants are proteinaceous competitive inhibitors acting as pseudo-substrates to enter the active site of target proteases. Following inhibition, the proteases can no longer cleave peptide bonds, thereby resulting in a detrimental inhibition of extracellular protein digestive functions in herbivores leading to significant growth and development delays (Arai *et al.*, 2002; Birk, 2003; Haq *et al.*, 2004).

Despite promising developments, the expression of recombinant protease inhibitors in plants raises a number of questions about the occurrence of unintended metabolic interference (or pleiotropic) effects on endogenous proteolysis eventually altering growth, development or chemical composition of the host-plant tissues. Proteolytic enzymes are ubiquitous effectors in living cells involved in the regulation of numerous metabolic processes, ranging from housekeeping functions such as protein turnover and the elimination of misfolded polypeptides to the processing of polypeptide pre- and pro-regions on maturing protein backbones (Estelle, 2001; Schaller, 2004; Smalle and Vierstra, 2004; Faye *et al.*, 2005; van der Hoorn, 2008). Whereas studies reported negligible phenotypic effects for protease inhibitors expressed in transgenic plants based on the assessment of macroscopic indicators such as growth rate, stem diameter or leaf number (e.g. Masoud *et al.*, 1993; Brunelle *et al.*, 2004; Rivard *et al.*, 2006; Badri *et al.*, 2009), recent reports suggest the onset of more subtle effects at the metabolic level. For instance, several studies documented the pesticidal effects of recombinant cystatins expressed in transgenic plants (e.g. Leplé *et al.*, 1995; Atkinson *et al.*, 2004; Outchkourov *et al.*, 2004), but the constitutive accumulation of these proteins *in planta* could also be associated with an alteration of flower development (Gutiérrez-Campos *et al.*, 2001), an inhibition of the hypersensitive response (Belenghi *et al.*, 2003), a modified physiological behaviour of the host plant under low temperature regimes (Van der Vyver *et al.*, 2003), or an altered total protein content in leaves (Van der Vyver *et al.*, 2003; Prins *et al.*, 2008). In a similar way, no visible phenotypic effects were observed for the Ser protease inhibitors bovine aprotinin and tomato Kunitz cathepsin D inhibitor (*S/CDI*) expressed in transgenic potato lines (Brunelle *et al.*, 2004; Rivard *et al.*, 2006), but metabolic interference significantly altering leaf protein levels were observed for both inhibitors (Badri *et al.*, 2009; Goulet *et al.*, 2009).

To further document the pleiotropic effects of recombinant protease inhibitors in plants and to assess their possible impact on the composition and authenticity of food products, we examined the chemical composition and proteome profile of potato tubers harvested from a collection of *S/CDI*-expressing, transgenic potato lines (Brunelle *et al.*, 2004; Rivard *et al.*, 2006). A classical scheme for the safety assessment of foods derived from transgenic crops consists of comparing their chemical composition with the composition of a conventional counterpart referred to as the comparator line, preferentially derived from a sister

line or the parental line used for genetic transformation (OECD, 1993; George *et al.*, 2004). Based on this, a transgenic product is considered as likely safe for human use if it is found to be 'substantially equivalent' to a conventional comparator with a documented history of safe use, provided that the encoded recombinant protein is not allergenic, antinutritive or toxic to eventual consumers (OECD, 2002; El Sanhoty *et al.*, 2004). For potato, about 30 chemicals have been identified as key nutritional or toxic indicators for the assessment of substantial equivalence among tubers, including proximate components such as total solids, total sugars, total proteins, total lipids, ash and fibres; and specific nutrients or toxic compounds such as sucrose, glucose, starch, vitamin C, amino acids, potassium, magnesium, copper and glycoalkaloids (AOAC, 2000a; OECD, 2002). Detailed studies have been published in recent years comparing the chemical composition of transgenic potato tubers with the composition of a conventional comparator (Rogan *et al.*, 2000; El Sanhoty *et al.*, 2004; Shepherd *et al.*, 2006). In this study, we combined this approach with a detailed proteomic profiling of tuber proteins (Lehesranta *et al.*, 2005; Bauw *et al.*, 2006) to compare in an integrated way the composition of tubers harvested from different *S/CDI*-expressing and comparator potato lines. We recently observed a significant impact of *S/CDI* on the leaf proteome of potato lines constitutively expressing this protein (Goulet *et al.*, 2009), which raised questions about the chemical composition of tuber tissues in these plants and suggested their possible usefulness as models for the validation of safety assessment strategies adapted to food products derived from novel-generation transgenic plants.

Results

S/CDI-expressing and control potato tubers are substantially equivalent

Seven *S/CDI*-expressing potato lines exhibiting variable transgene expression and *S/CDI* protein levels in leaves were selected for the compositional analyses ('CD' lines in Rivard *et al.*, 2006; Goulet *et al.*, 2009), along with three comparator lines consisting of the parental line K and two transgenic control lines ('SPCD' lines) bearing the *slcdi* transgene with no promoter for heterologous expression. PCR assays were first performed to confirm the presence of the *nptII* (selection marker) and *slcdi* transgenes in tubers of the nine transgenic lines, and to confirm the absence of *slcdi* mRNA transcripts in control lines

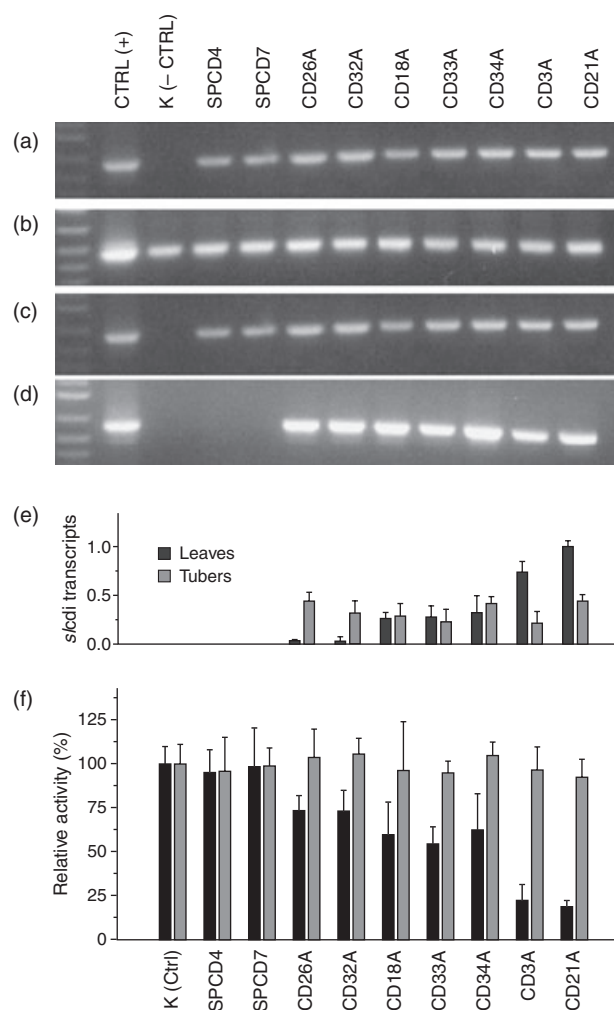


Figure 1 Integration and expression of the tomato cathepsin D inhibitor (*slcdi*) transgene in tubers of transgenic potato lines. (a–c) Polymerase chain reaction (PCR) detection of the *nptII* marker transgene (a), endogenous *S/CDI*-like gene(s) (b) and *slcdi* transgene (c) in control (K, SPCD) and *S/CDI*-expressing (CD) lines. (d, e) Reverse transcriptase (RT)-PCR detection of *slcdi* mRNA transcripts in control and CD lines. (f) Trypsin inhibitory activity in leaf and tuber protein extracts of control and CD lines. DNA amplicons following PCR and RT-PCR were submitted to agarose gel electrophoresis and visualized by staining with ethidium bromide. Amplicons produced from DNA templates were used as positive controls for the tests [CTRL(+)]. Data in (e) are expressed as relative amounts of *slcdi* transcripts compared with the amount in leaves of clone CD21A (arbitrary value of 1.0). Data in (f) are expressed as relative activities compared with the activity in leaf or tuber protein extracts of control line K. Each bar on panels (e) and (f) is the mean of three independent (biological) replicates $\pm$ SE.

(Figure 1). As expected, the *nptII* transgene was amplified from total DNA extracts of all CD and SPCD lines, unlike nontransgenic line K showing no positive signal (Figure 1a). Likewise, the *slcdi* transgene was present in all CD and SPCD lines but absent from the parental line (Figure 1c), in contrast with (an) endogenous CDI-encod-

ing gene(s) amplified in all lines using potato-specific oligonucleotide primers (Figure 1b). In agreement with the absence of a promoter sequence in gene constructs, no *slcdi* transcript signal was amplified from the SPCD lines by reverse transcriptase (RT)-PCR, similar to parental line K (Figure 1d,e). By comparison, *slcdi* transcripts were easily detected in the seven CD lines, albeit at variable levels from one line to another. Unlike leaf protein extracts, tuber protein extracts from the *S/CDI*-expressing lines had no measurable trypsin inhibitory activity *in vitro* against bovine trypsin used as a target (Figure 1f), which suggests either a very low abundance of *S/CDI* in tuber tissues and/or a negligible functional impact of this protein in the presence of highly abundant endogenous trypsin inhibitors.

A comparative compositional analysis was performed with the ten lines selected to address this question, using as source material 5 cm-long tubers from 4 month-old plants acclimated and grown in greenhouse. In brief, no significant difference was observed among the lines for a number of proximate components including total solids, total proteins, total lipids, total sugars, ash and crude fibres (Table 1). As well, no difference was observed for key nutritional indicators of potato tubers such as glucose, sucrose, starch, soluble proteins, vitamin C, amino acids and selected minerals (Tables 2 and 3), except for magnesium levels showing slight but significant variations in one control (SPCD4) and two *S/CDI*-expressing (CD26A and CD21A) lines (ANOVA: $P < 0.05$). Overall, these data showing a similar chemical composition for the different tubers, together with the absence of glycoalkaloids in both control and *S/CDI*-expressing lines (Table 2), indicate that tubers harvested from *S/CDI*-expressing and comparator lines were substantially equivalent despite detectable expression of the *slcdi* transgene in tuber RNA extracts (Figure 1d,e), measurable *S/CDI* inhibitory activity in leaves (Figure 1f), and significant interference of *S/CDI* on the overall proteome of leaf tissues (Goulet *et al.*, 2009).

Slcdi transgene expression has no significant impact on the tuber proteome

A proteomic analysis was carried out to determine whether the null impact of *S/CDI* on tuber composition could be associated with a negligible amount of this protein *in planta*, and to look for eventual minor pleiotropic effects detectable at the proteome level. To this end we compared the two-dimensional gel electrophoresis (2-DE) protein profiles of tubers harvested from two SPCD

Table 1 Proximate chemical composition of tubers from control and tomato cathepsin D inhibitor (*S/CDI*)-expressing potato lines

Component	Concentration (% dry weight)*									
	K (ctrl)	Transgenic controls		Transgenic test lines						
		SPCD4	SPCD7	CD26A	CD32A	CD18A	CD33A	CD34A	CD3A	CD21A
Total solids	20.5	20.2	21.0	21.0	20.8	20.7	21.4	20.6	20.5	22.2
Total proteins	9.92	9.63	9.46	9.44	9.32	9.61	9.31	9.73	9.92	9.11
Total lipids	1.04	1.14	0.95	1.05	1.10	1.11	1.03	0.97	0.98	1.04
Total sugars	77.9	78.3	78.8	78.8	78.0	78.4	79.1	78.1	77.8	79.7
Ash	4.48	4.52	4.37	4.34	4.42	4.35	4.26	4.43	4.49	4.15
Crude fibre	1.22	1.29	1.28	1.29	1.25	1.21	1.22	1.26	1.27	1.22

*Data are presented as mean contents. No significant difference was observed among the ten lines for all components assessed (ANOVA; $P > 0.05$).

Table 2 Selected nutrients and antinutritional compounds in tubers of control and tomato cathepsin D inhibitor (*S/CDI*)-expressing potato lines

Component	Concentration (% fresh weight)*									
	K (ctrl)	Transgenic controls		Transgenic test lines						
		SPCD4	SPCD7	CD26A	CD32A	CD18A	CD33A	CD34A	CD3A	CD21A
Glucose	0.12	0.13	0.12	0.12	0.13	0.13	0.11	0.12	0.12	0.12
Sucrose	0.20	0.20	0.21	0.21	0.18	0.21	0.21	0.21	0.20	0.21
Starch	14.7	14.4	14.3	14.8	14.7	14.5	14.6	14.6	14.8	14.6
Soluble proteins	0.61	0.71	0.66	0.63	0.63	0.71	0.73	0.71	0.72	0.79
Vitamin C†	79.1	79.0	79.2	78.9	79.3	79.8	79.0	79.5	79.2	77.9
Glycoalkaloids‡	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

*Data are presented as mean contents. No significant difference was observed among the ten lines for all components assessed (ANOVA; $P > 0.05$).

†Expressed in mg/100 g fresh weight.

‡ND, not detected (limit of detection: 0.1 mg/100 g fresh weight).

comparator lines, lines SPCD4 and SPCD7, with the 2-DE tuber protein profiles of two *S/CDI*-expressing lines, lines CD3A and CD21A (Figure 2), exhibiting *S/CDI* levels in leaves estimated at ~0.1% of total soluble proteins (Goulet *et al.*, 2009), comparable with expression levels reported earlier for insect-resistant transgenic tobacco lines expressing Ser-type inhibitors (Hilder *et al.*, 1987; Johnson *et al.*, 1989). Because most proteins in tuber extracts exhibited a neutral or mildly-acidic *pI* value (Figure 2a), a 4–7 pH gradient was used for isoelectric focusing (IEF) to minimize physical interference during migration or masking effects upon staining due to the presence of highly abundant proteins (Figure 2b).

In brief, most of the 550–600 proteins detected in 2-D gels after colloidal blue staining were found at similar levels in the four lines tested, including multiple components of the major potato storage protein, patatin. By contrast,

21 proteins showed a significantly altered level in at least one line (Figure 2b). Protein spots #3 and 12, for instance, were less abundant in lines SPCD7 and CD3A than in lines SPCD4 and CD21A, compared with protein spots #1, 7 and 18 being less abundant in line SPCD4 than in the three other lines (Figure 3). Despite significant differences, none of the proteins showing variation did correspond to abundant (e.g. storage) proteins, and most alterations fell within a less than twofold variation range. Most importantly, no variation measured in the 2-D gels could be correlated with the presence or absence of the *slcdi* transgene. Several proteins, for instance, were found at different levels in the two SPCD lines or followed diverging variation patterns in the CD lines (Figure 3), which might indicate somaclonal variation or false-positive alterations following ANOVA, within the error range of 5% adopted for statistical significance. Possibly accounting for the

Table 3 Selected minerals and amino acids in tubers of control and tomato cathepsin D inhibitor (*S/CDI*)-expressing potato lines

	Concentration (mg/100 g fresh weight)*									
		Transgenic controls		Transgenic test lines						
Component	K (ctrl)	SPCD4	SPCD7	CD26A	CD32A	CD18A	CD33A	CD34A	CD3A	CD21A
Minerals										
Potassium	326	340	348	385	303	329	381	342	388	388
Magnesium	17.6	22.6*	20.7	21.5*	20.3	19.8	20.6	20.0	20.5	23.5*
Copper	0.14	0.18	0.22	0.22	0.23	0.18	0.19	0.24	0.17	0.18
Amino acids										
Aspartate	30.4	34.0	34.0	35.0	30.7	27.8	32.7	33.0	30.6	34.7
Methionine	14.2	14.2	20.3	18.8	20.3	14.2	21.3	16.2	21.3	16.2
Glutamate	7.85	8.11	9.63	9.93	9.60	7.09	7.19	9.47	8.41	8.71
Asparagine	11.0	10.8	11.7	10.2	10.7	10.4	9.53	10.9	10.9	10.4
Glutamine	535	538	622	598	553	469	551	631	572	664
Cysteine	33.4	31.4	30.4	36.5	36.0	34.5	35.5	35.5	33.4	34.0
Glycine	3.55	3.50	4.05	2.52	2.53	3.54	3.04	2.50	2.51	2.03
Histidine	244	255	258	205	209	211	247	230	238	242
Phenylalanine	8.61	10.1	10.6	9.12	7.09	9.60	9.63	7.60	8.11	6.59
Serine	3.55	4.56	6.08	6.08	6.08	6.59	7.60	5.07	8.11	7.60
Threonine	7.09	8.60	8.59	8.59	8.11	10.6	6.59	7.60	10.1	9.12
Tryptophan	713	648	694	727	746	702	735	710	683	695
Tyrosine	72.0	70.4	74.0	69.9	58.8	73.0	68.9	68.4	69.9	73.0
Arginine	116	99.3	90.7	106	105	115	115	98.8	86.1	94.8

*Data are presented as mean concentrations. No significant difference was observed among the ten lines for all components assessed (ANOVA; $P > 0.05$), except for magnesium showing a significant difference in some transgenic control or test lines ($*P < 0.05$) compared with untransformed mother line K.

negligible impact of *slcdi* transgene expression, no protein spot was detected in 2-D gels for the two CD lines at the 22.0 kDa/pI 8.9 intersect corresponding to the expected migration coordinates of recombinant, nonglycosylated *S/CDI*. In agreement with compositional data indicating substantial equivalence among tubers of test and control lines (see above), these observations suggest, overall, a negligible effect of *slcdi* at the proteome level associated with a very low abundance of *S/CDI* in tuber cells despite the presence of mRNA transcripts in tuber RNA preparations (Figure 1e) and the detection of active *S/CDI* in leaves (Figure 1f).

***Slcdi* transgene expression has no impact on the deposition pattern of protein allergens**

Immunodetection assays were carried out to formally confirm the negligible abundance of *S/CDI* in tuber protein extracts and to assess the overall deposition pattern of endogenous Kunitz protease inhibitors (Pouvreau *et al.*, 2001; Heibges *et al.*, 2003), identified earlier as potential protein allergens in potato tubers along with the multifunctional storage protein patatin (Seppälä *et al.*, 2001).

We observed above a conserved deposition pattern for components of the patatin complex in tubers of control and *S/CDI*-expressing lines (see Figure 2). As a complement, we immunodetected and assayed Kunitz inhibitors in tuber extracts, after designing and producing chicken polyclonal antibodies for the probing of a 15-aa loop protruding at the surface of *S/CDI* and potato Kunitz inhibitors (see Figure 4a). Four bands c. 17–23 kDa in size were detected on immunoblots for all lines tested, including control parental line K (Figure 4b). No extra signal was detected for *S/CDI* in tubers of the CD lines, in contrast with (healthy) leaves of line CD3A exhibiting a ~20-kDa protein undetected in line K, immunologically related to bacterially expressed *S/CDI* (Figure 4c). The detection of a unique, multiprotein pattern in test and control lines, along with the absence of an extra signal for *S/CDI* in the CD lines, again suggested the very low abundance of *S/CDI* in transgenic tubers (see above) and a null impact for this protein on the deposition of endogenous Kunitz proteins.

A 2-D immunoblot study was conducted to confirm in more detail the null impact of *slcdi* transgene expression on Kunitz proteins (Figure 5). The tuber proteins of lines

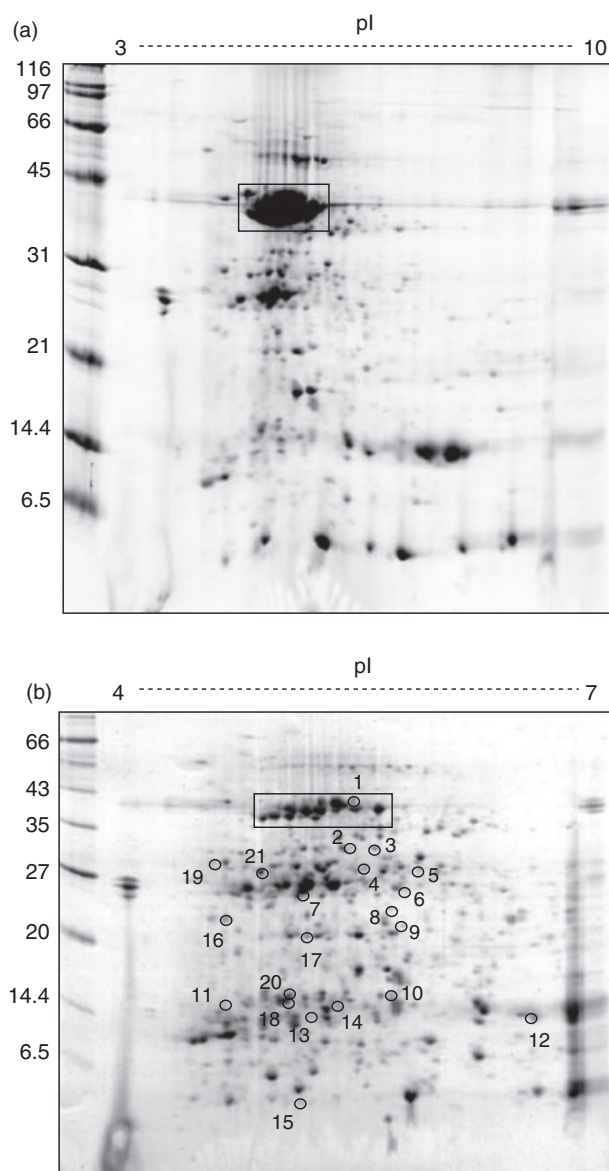


Figure 2 Representative two-dimensional gel electrophoresis protein profiles for tubers of transgenic control line SPCD4. Isoelectric focusing for the 1st dimension was performed along 3–10 (a) or 4–7 (b) pH gradients. About 550–600 protein spots were observed on gels after colloidal blue staining. Numbered spots on panel (b) refer to proteins showing altered levels in at least one line (see Figure 3 for quantitative details). Square boxes on panels (a) and (b) point to multiple components of the patatin storage protein complex (see Bauw *et al.*, 2006). Numbers and protein scales on the left correspond to commercial molecular weight markers (kDa).

SPCD4, SPCD7, CD3A and CD21A were first resolved in 2-D polyacrylamide slab gels as described above (Figure 5a), and then transferred onto nitrocellulose sheets for immunodetection with the anti-*S/CDI* loop polyclonal antibodies. About 25 proteins were immunodetected for the different lines (Figure 5b), in accordance with the

occurrence of multi-member Kunitz protein families in potato (Heibges *et al.*, 2003). Most of these proteins showed a neutral or basic pI and a molecular weight in the c. 16–25-kDa range (Figure 5b) typical of Kunitz protease inhibitors (Suh *et al.*, 1990, 1991; Kreft *et al.*, 1997; Pouvreau *et al.*, 2001; Kang *et al.*, 2002; Heibges *et al.*, 2003). Protein spots matching the immunodetected proteins in Coomassie blue-stained template gels (Figure 5a) were found at comparable levels in all lines tested (ANOVA; $P > 0.05$), thus confirming the null impact of the *slcdi* transgene on the overall pattern of proteins immunologically related to *S/CDI*. Several of these proteins were successfully identified by mass spectrometry (MS) (Figure 5a,b, spots A–M) and shown to belong to the Kunitz family of protease inhibitors, as expected (Tables 4 and S1). Except for abundant isoforms of the structurally distinct proteinase inhibitor II (spots A–D), all identified proteins included in their primary sequence a hydrophilic amino acid sequence highly similar to the *S/CDI* loop targeted by the chicken antibodies (Table 4). In agreement with total protein 2-DE analyses (see above), no specific signal could be detected for *S/CDI* in *S/CDI*-expressing potatoes, in sharp contrast with five endogenous CDI homologues easily detected in both control and CD lines (Figure 5 and Table 4, spots G–K). These observations confirming the absence of detectable *S/CDI* in tuber extracts of lines CD3A and CD21A, along with the identical protein profiles observed for patatin and Kunitz inhibitors in CD and SPCD lines, unequivocally indicated a negligible impact of *slcdi* transgene expression on the deposition pattern of potential protein allergens in tubers of *S/CDI*-expressing potatoes.

Discussion

High-throughput profiling technologies such as transcriptomics, proteomics and metabolomics present a number of advantages over classical compositional studies for the characterization of transgenic plant lines and foods derived from transgenic crops (Kuiper *et al.*, 2003; Rischer and Oksman-Caldentey, 2006; Kok *et al.*, 2008; Davies, 2009; Miki *et al.*, 2009), including a dramatically improved resolution power allowing for the simultaneous analysis of hundreds or thousands of chemical components, or 'characters'. From a regulatory or safety monitoring viewpoint, these technologies make it possible not only to assess a defined number of well-characterized nutritional indicators selected *a priori*, but also to detect uncharacterized (and even unknown) factors eventually affected *in planta* by

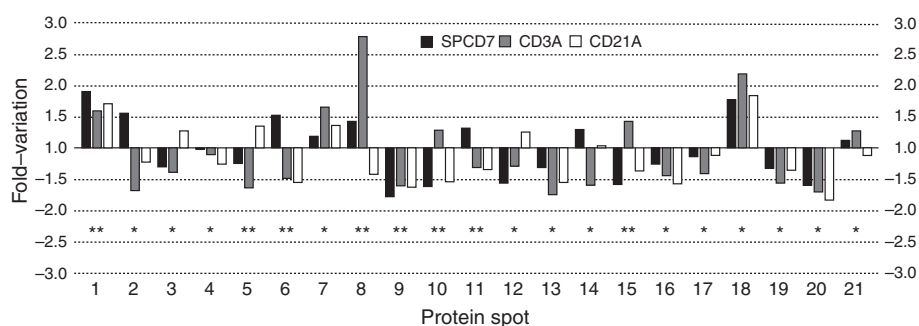


Figure 3 Relative levels of protein spots exhibiting altered levels among control and test lines (see Figure 2b for spot numbering). Data are expressed as relative variations in lines SPCD7, CD3A and CD21A compared with line SPCD4 (arbitrary value of 1.0). Positive (negative) values indicate higher (lower) relative levels compared with line SPCD4. Asterisks indicate significant (*) or highly significant (**) variations among the four lines assessed (ANOVA; $P < 0.05$ or $P < 0.01$).

the insertion or expression of the transgene. As importantly, they allow for the *a posteriori* identification and characterization of any unintended effect using appropriate gene sequencing, chromatographic or mass spectrometric methods, thereby simplifying data interpretation in terms of biological effects and significance. Proteomic approaches, in particular, appear of obvious interest to detect and monitor the unintended or pleiotropic effects of a transgene or recombinant protein *in planta* (Corpillo *et al.*, 2004; Lehesranta *et al.*, 2005; Ruebelt *et al.*, 2006a,b,c; Lovegrove *et al.*, 2009), given the central structural and functional roles assigned to proteins in plant cells and tissues. In the present study we followed an integrated, dual evaluation scheme to assess the impact of *S/CDI* on the chemical composition of tubers harvested from *S/CDI*-expressing transgenic potato lines, first involving a compositional analysis of key nutrients, and then the monitoring of tuber proteome profiles.

S/CDI constitutively expressed in transgenic lines of potato was shown recently to significantly change the overall proteome profile in leaves via an unelucidated proteome-wide alteration of the protein synthesis/turnover balance (Goulet *et al.*, 2009). In sharp contrast with such dramatic effects on leaf protein catabolism, we observed here no impact for *S/CDI* on both the nutritional composition and general proteomic profile of *S/CDI*-expressing tubers. Small significant variations were noted for magnesium among SPCD and CD tuber extracts, but mean levels fell, in all lines, within the concentration range naturally observed for this element in tubers of commercial potato varieties (Leszczynski, 1989). In a similar way, a number of significant changes were detected at the proteome level, but no formal link could be made with the presence or expression of the tomato transgene in *S/CDI*-expressing lines. These observations suggest, overall, a negligible

impact of *S/CDI* expression on tuber composition, and the onset of interline somaclonal or epigenetic variations among test and control lines possibly induced during genetic transformation or plantlet regeneration (Jongedijk *et al.*, 1992; Kawchuk *et al.*, 1997).

Complementary 2-DE analyses were carried out to visualize the deposition pattern of endogenous Kunitz protease inhibitors, abundant in tuber tissues (Pouvreau *et al.*, 2001; Heibges *et al.*, 2003) and identified earlier as potential allergens in potato tubers, along with multiple components of the patatin protein complex (Seppälä *et al.*, 2001). Empirical analysis of the host-plant allergenic potential still represents a key step in the overall safety evaluation of transgenic food products (Kok *et al.*, 2008), as there is yet no logical scheme to predict *in silico*, with a high degree of confidence, the impact of transgene integration or recombinant protein expression on the deposition pattern of endogenous allergens (FAO/WHO, 2001; Codex Alimentarius Commission, 2003; Kleter and Peijnenburg, 2004; Sanchez-Monge and Salcedo, 2005). Such an analysis was of particular relevance in the present case given the high structural homology between *S/CDI* and potato Kunitz inhibitors, readily recognized as belonging to the same protein family (Werner *et al.*, 1993). In summary, our data indicated no significant difference among *S/CDI*-expressing and control tubers for the deposition pattern of Kunitz-type proteins in tubers, similar to patatin subunits showing unaltered levels in 2-D gels following colloidal Coomassie blue staining. Along with data obtained for the general proteome profile, these observations supported, overall, the conclusions of our compositional data showing substantial equivalence between comparator and *S/CDI*-expressing tubers. They also unequivocally confirmed the very low abundance of *S/CDI* in tuber tissues, which in turn likely accounted for

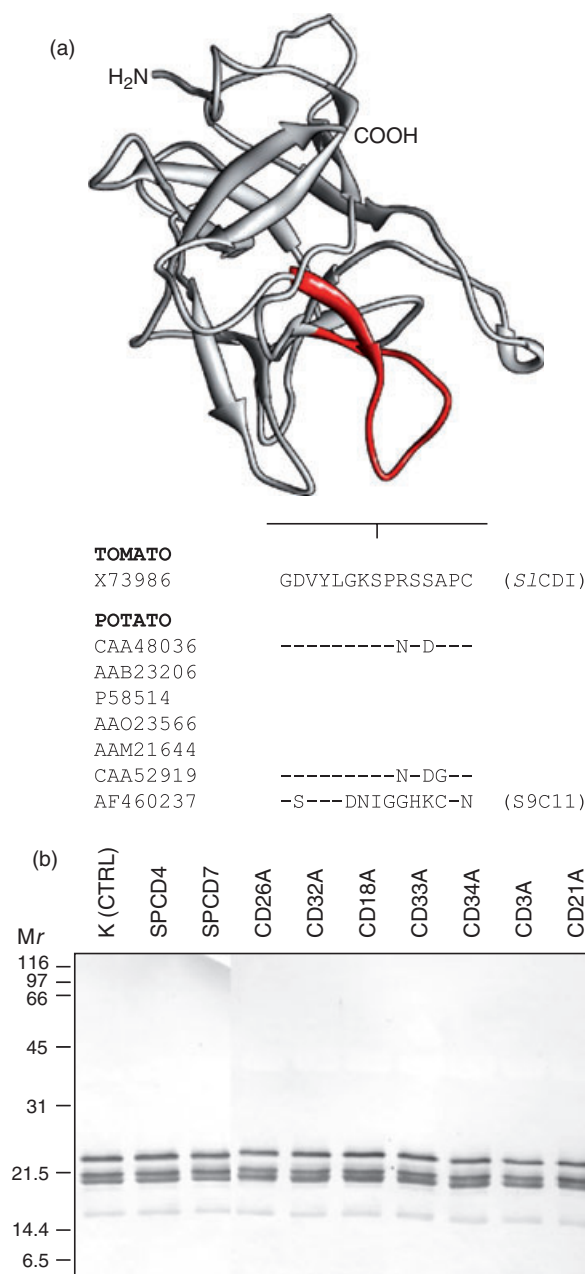


Figure 4 Immunodetection of tomato cathepsin D inhibitor (*S/CDI*) and endogenous Kunitz protease inhibitors in leaf and tuber protein extracts of control and *S/CDI*-expressing potato lines. (a) Tertiary structure model for *S/CDI* showing the position and spatial orientation of a 15-aa hydrophilic surface loop targeted for the production of anti-*S/CDI* polyclonal antibodies (in red) (see Experimental procedures for details). A sequence alignment generated with CLUSTAL W (Higgins *et al.*, 1994) compares the loop sequence of *S/CDI* with the corresponding loop of selected potato Kunitz proteins including S9C11, a distant homologue down-regulated in leaves of *S/CDI*-expressing potato lines (see Goulet *et al.*, 2009). Alphanumeric codes refer to NCBI GenBank accession numbers (<http://www.ncbi.nlm.nih.gov>). The ‘—’ signs indicate identity with corresponding residues in *S/CDI*. (b) Immunodetection of *S/CDI* and potato Kunitz inhibitors in tuber protein extracts using the anti-*S/CDI* loop polyclonal antibodies. (c) Immunodetection of *S/CDI* in leaf protein extracts of lines K and CD3A using the same antibodies. r*S/CDI*, nonglycosylated *S/CDI* expressed in *Escherichia coli* (Brunelle *et al.*, 2005). Numbers on the left of panels (b) and (c) refer to molecular weight markers (kDa).

the negligible effects of *slcdi* transgene expression on tuber composition.

It is not clear, at this point, why *S/CDI* could not be detected in tuber extracts of lines CD3A and CD21A despite the presence of *slcdi* transcripts at levels roughly comparable to those measured in leaves and the detection of biologically active *S/CDI* in leaf tissues of both two lines. One hypothesis could involve the variable (in)stability and turnover rate of *S/CDI* in cellular environments harbouring distinct proteolytic machineries, as discussed earlier for a number of recombinant proteins in plant systems (Benchabane *et al.*, 2008). An alternative hypothesis

would be the natural sink effect of highly abundant storage proteins on amino acid resources, inherently unfavourable to the biosynthesis and accumulation of transgene-encoded proteins (Schmidt and Herman, 2008). We recently established a link between *S/CDI* biosynthesis in potato leaves and the down-regulation of its endogenous homologue, S9C11 (Goulet *et al.*, 2009). By contrast, *S/CDI* accumulation could have been compromised in tubers by highly expressed *S/CDI* homologues strongly attracting amino acids at the mRNA translation stage. Although protein accumulation in storage organs is influenced by mineral nutrition and some other environmental

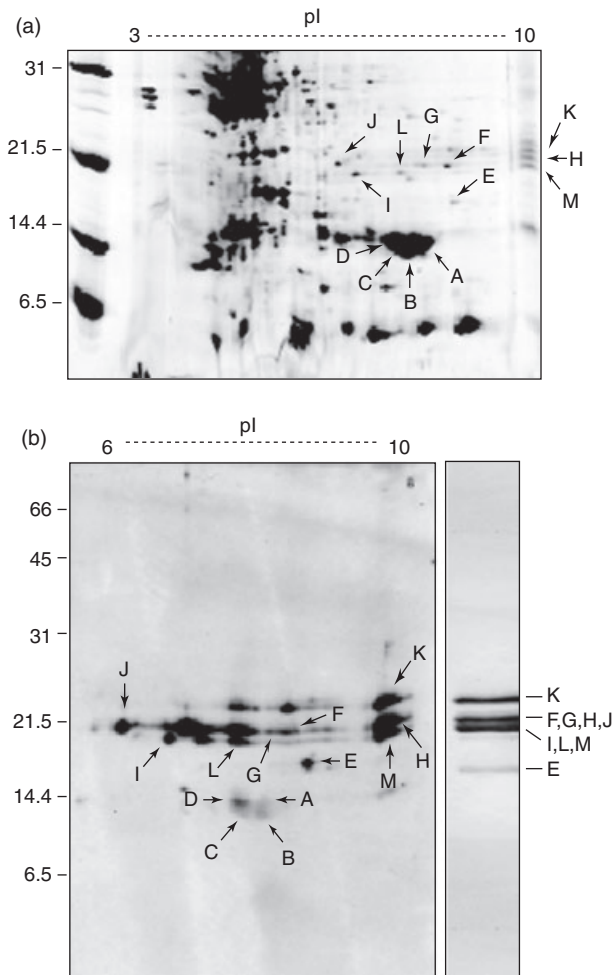


Figure 5 Electrophoretic separation and immunodetection of Kunitz protease inhibitors in a tuber protein extract of control line SPCD4. (a) Colloidal blue-stained proteins resolved by two-dimensional gel electrophoresis (2-DE). Protein bands and numbers on the left correspond to molecular weight markers (kDa). (b) Immunodetection of tomato cathepsin D inhibitor (*SlCDI*) and immunologically related (Kunitz-type) tuber proteins following 2-DE (left panel) or sodium dodecyl sulphate–polyacrylamide gel electrophoresis (right panel). Polyclonal antibodies raised against the 15-aa hydrophilic surface loop of *SlCDI* were used as primary antibodies (see Figure 4, above). Letters A to M refer to proteins identified by LC-MS/MS (see Tables 4 and S1). A 3–10 pH gradient was used for isoelectric focusing. Numbers on the left indicate the position of commercial molecular weight markers (kDa).

factors (Tabe *et al.*, 2002), the relative distribution of proteins in storage tissues exhibits a very limited plasticity and is, for the most part, genetically determined (Schmidt and Herman, 2008). Studies reported the potential of low storage protein mutants (Tada *et al.*, 2003) or transgenic host plants deficient in major storage proteins (Goossens *et al.*, 1999; Kim *et al.*, 2008; Schmidt and Herman, 2008) as efficient production platforms for foreign proteins, thereby suggesting both the natural intrinsic inertia of the storage organ proteome and the practical relevance of redirecting

the host-plant endogenous protein biosynthesis machinery to the expression of foreign proteins for applications such as molecular farming or nutritional engineering involving recombinant protein deposition. This limited plasticity of the proteome in storage organs could represent, on the other hand, an advantage from a regulatory or safety viewpoint for biotechnological applications targeted to nonstorage organs, especially for those proteins such as *SlCDI* exhibiting high homology with endogenous (and potentially allergenic) storage proteins. Work is underway to test this idea, using a number of recombinant proteins and promoter sequences expressed in different organs of potato.

Experimental procedures

Potato lines and tuber processing

Seven transgenic lines of potato (*Solanum tuberosum* L., cv. Kennebec) expressing *SlCDI* under the control of the cauliflower mosaic virus 35S constitutive promoter were used as source material for the experiments (CD lines), along with two transgenic sister lines bearing the *slcdi* transgene with no promoter (SPCD lines) and *in vitro* clones of control parental line K used for genetic transformation (Brunelle *et al.*, 2004). These 10 lines exhibit different levels of *slcdi* mRNA transcripts in leaves, ranging from null or barely detectable in untransformed and transgenic controls to moderate or high in transgenic test lines (see Figure 1a). At least three tubers of each line were sown and grown for four months in greenhouse at the Centre de recherche en horticulture of Laval University (Québec, QC, Canada). Three to five tubers of similar size (ca. 5 cm long) were harvested in each pot and used for chemical and molecular analyses. The tubers were washed, peeled, sliced and ground to a fine powder in liquid nitrogen. The powder was lyophilized to dryness and stored at -80°C until use.

Insertion of the *nptII* and *slcdi* transgenes

Stable insertion of the *nptII* gene selection marker (see Brunelle *et al.*, 2004) and *slcdi* transgenes was confirmed by PCR amplification from genomic DNA samples using appropriate oligonucleotide primers. Genomic DNA was extracted essentially as described by Hwang and Kim (2000). In brief, 250 mg of tuber powder was mixed with 300 μL of modified CTAB buffer (mCTAB) consisting of 2% (w/v) CTAB (Sigma-Aldrich, Oakville, ON, Canada), 1.4 M NaCl, 100 mM Tris-HCl (pH 8.0), 50 mM ethylenediamine tetraacetic acid (EDTA), 1% (w/v) PVP40 (Sigma-Aldrich) 0.5% (w/v) sodium bisulfide and 1% (v/v) 2-mercaptoethanol, and incubated with gentle agitation at 65°C for 20 min. Three hundred microlitres of chloroform was added to each sample, the tubes were vortexed for 10 s at maximal speed, and centrifuged at 12 000 *g* for 5 min at room temperature. The upper phase (containing DNA) was transferred in a sterile tube, and DNA was

Table 4 Proteins immunodetected in potato tuber protein extracts using chicken polyclonal antibodies directed against the 15-aa surface loop of tomato cathepsin D inhibitor (*S/CDI*) and potato Kunitz inhibitors (these proteins were detected at statistically similar levels in control and *S/CDI*-expressing lines*)

Putative protein	Accession no.	Spot no.†	Mr estim./calc.	pI estim./calc.	Sequence coverage (%)	Hydrophilic loop motif
<i>S/CDI</i>	–	–‡	–	–	–	GDVYLKGKSPRSSAPC§
Serine proteinase inhibitor I (PSPI-21)	P58514	E	18.4/16.3	8.5/5.0	18	-----N-D---
Cathepsin D inhibitor	S10721	G	21.3/20.8	8.0/7.8	56	-----N-D---
	P58521	H	21.7/18.9	9.8/9.2	56	
Aspartic proteinase inhibitor	CAA45723	I	20.6/24.1	6.8/7.5	20	-----N-D---
	AAB23206	J	21.5/24.6	6.5/6.3	43	
	P58518	K	22.3/20.5	9.8/8.6	60	
Proteinase inhibitor	BAA04152	L	20.7/23.5	7.6/6.8	12	-----N-D---
		M	21.0/23.5	9.8/6.7	51	
Proteinase inhibitor II (Pin II)	CAA27730	A	14.4/17.5	8.1/6.8	59	–CN–YSANGAFICEG
		B	14.2/17.5	7.8/6.8	60	
		C	14.3/17.5	7.6/6.8	50	
		D	14.5/17.5	7.4/6.8	55	

*Protein sequences retrieved from the NCBI GenBank database (<http://www.ncbi.nlm.nih.gov>). Sequence details for LC-MS/MS identifications are given online in Table S1.

†See arrows on Figure 5.

‡No *S/CDI* was detected in the potato lines analyzed.

§(–) indicate identity with the corresponding residue in *S/CDI*.

precipitated with an equal volume of isopropanol for 5 min at room temperature. After centrifugation for 5 min at 12 000 *g*, the pellet was Speedvac-dried and resuspended in 50 µL of sterile TE buffer containing RNase A (50 µg/mL), before measuring A_{260}/A_{280} ratios using a UV spectrophotometer to monitor the quality of the DNA samples. The *nptII* and *slcdi* transgenes were detected using the following primers: 5'-ACTGA AGCGG GAAGG GACTG GCTGC TATTG and 3'-GATAC CGTAA AGCAC GAGGA AGCGG TCAG for *nptII*; and 5'-AAGGA TCCGT GCACA AAAGA TGGCT GCTTC TAAAC CTAAT CCAGT AC and 3'-AACCC GGGAA GCCGA GACTT TCTTG AAGTA GACCC CCAAG for *slcdi*. Potato endogenous CDI-encoding gene sequences were monitored as a control, using the following degenerated primers: 5'-AGGAT CGGTG MWTCT CCTMW ACCTA AKCCR GTAC and 3'-AGAAT TCTAG CTAGA CTTC TTGAA GTTGA C, where M = A or C, W = A or T, K = G or T and R = G or A (Brunelle *et al.*, 2005). After PCR, the amplicons were visualized by ethidium bromide staining following 1% (w/v) agarose gel electrophoresis.

Expression of the *slcdi* transgene

Expression of the *slcdi* transgene was monitored by RT-PCR, using tuber total RNA samples and oligonucleotide primers for *slcdi* amplification (see above). Total RNA was extracted from tuber powder using the Concert Plant RNA Reagent from Invitrogen Life Technologies (Burlington ON, Canada), following the supplier's instructions. After precipitation in isopropanol, total RNA was washed in 75% (v/v) ethanol, dissolved in RNase-free water, and proofchecked for quality by the monitoring of A_{260}/A_{280} and A_{260}/A_{230} absorbance ratios. cDNA populations were synthesized

using the Superscript kit for cDNA synthesis (Invitrogen), and used as templates for PCR. The PCR amplicons were resolved by 1% (w/v) agarose gel electrophoresis and visualized by ethidium bromide staining. For quantitation, the gels were scanned using an Amersham Image Scanner digitalizer (GE Healthcare, Baie d'Urfé, QC, Canada) prior to computer processing and image analysis. Image analysis was carried out as described recently (Badri *et al.*, 2009), using the PHORETIX 2D EXPRESSION software, v. 2005 (NonLinear USA, Durham, NC, USA). Three biological (tuber) replicates were used for each line to allow for statistical assessments (ANOVA; $\alpha = 0.05$).

S/CDI inhibitory activity

S/CDI protease inhibitory activity in leaf and tuber protein extracts was assayed *in vitro* using the colorimetric protein substrate azocasein and bovine trypsin as a target protease (Michaud and Vrain, 1998). Soluble proteins were extracted from tuber or leaf powder in 50 mM Tris-HCl, pH 7.5, containing 1 mM EDTA (see Rivard *et al.*, 2006). Trypsin activity was monitored in 50 mM Tris-HCl, pH 8.0, after adding 100 µL (~100 µg protein) of tuber or leaf extract from either *S/CDI*-expressing or control plants (see Michaud and Vrain, 1998). An arbitrary value of 100% residual activity was assigned to trypsin activities measured with leaf or tuber extracts of control line K.

Immunodetection of *S/CDI*

S/CDI in tuber and leaf extracts was probed by immunodetection with IgY primary antibodies generated in chicken by AgriSera

(Vännäs, Sweden), against the 15-aa peptide sequence 'GDVYLGKSPRSSAPC' forming a hydrophilic loop at the surface of the inhibitor (see below for *S/CDI* structure modelling). The proteins were resolved by 12% (w/v) or 15% (w/v) sodium dodecyl sulphate–polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose membranes for immunodetection. The membranes were saturated with 5% (w/v) skim milk in Tris-buffer saline containing 0.05% (v/v) Tween-20, washed three times for 10 min in Tris-buffer saline, and then incubated for 1 h at 22 °C with the anti-*S/CDI* IgY chicken antibodies. After washings in Tris buffer, the membranes were incubated for 1 h at 22 °C with goat anti-chicken secondary antibodies labelled with peroxidase, washed three times, and revealed by chemiluminescence using the Western Lightning immunodetection kit (Perkin Elmer Life Sciences, Waltham, MA, USA).

Tertiary structure modelling

A tertiary structure model for *S/CDI* was inferred based on the spatial coordinates of soybean Kunitz trypsin inhibitor [Protein Data Bank (<http://www.rcsb.org/pdb>), Accession no. 1AVU] (Song and Suh, 1998). The model was constructed using the software MODELLER, v. 6.2 (Sanchez and Sali, 2000), and tested for energy distribution and stereochemical quality using the in-built Energy command of MODELLER and the PROCHECK program (Laskowski *et al.*, 1993), respectively. *S/CDI* was visualized with the RASMOLE software (Sayle and Milner-White, 1995).

Compositional analysis

The chemical composition of potato tubers was assessed by a two-step approach first involving a proximate analysis to compare relative levels of major constituents such as total solids, total proteins, total lipids, total carbohydrates, ash and crude fibres; followed by the targeted analysis of specific nutritive and toxic compounds typically assessed for potato tubers (AOAC, 2007a; OECD, 2002). All measures were repeated three times with samples from distinct tubers. Data for each compound were analyzed using *F* statistics obtained from a one-way ANOVA, with plant line as the main effect. An error rate of 5% was used to interpret the results.

Proximate analysis

Total solids were determined by the estimation of tuber powder weight loss upon drying in an oven at 100 °C (AOAC, 2007a). Total proteins were determined from total nitrogen quantitation by the Kjeldahl method (AOAC, 2007b), using the multiplication factor 'N X 6.25' for crude protein content estimation. Fat content was determined by the Soxhlet extraction method (AOAC, 2007c) after tuber sample extraction in petroleum ether and extract evaporation, drying and weighing. Ash content was estimated by ignition to a constant weight in an electric furnace at 550 °C, and subsequent quantification of the residues (AOAC, 2007d). Total carbohydrates were estimated by difference using the following equation: carbohydrates (%) = 100% – (% protein + % fat + % ash + % moisture) (USDA, 1973). The content in crude fibres was determined following sample digestion in

1.25% (v/v) sulfuric acid and 1.25% (v/v) sodium hydroxide (AOAC, 2007e). Crude fibre content was estimated as residue weight loss after drying, weighting and ignition at 550 °C.

Ascorbic acid (vitamin C)

Ascorbic acid was extracted from lyophilized tuber samples and quantitated by high performance liquid chromatography (HPLC) under a UV detector as described by Spares *et al.* (1990). L-Ascorbic acid (Sigma-Aldrich) was used as a reference standard.

Starch and soluble sugars

Soluble sugars in tuber powder samples were extracted for 30 min at 80 °C in 80% (v/v) ethanol. After centrifugation for 5 min at 3000 g, the supernatant was recovered for glucose, fructose and sucrose enzymatic quantitation in the presence of ATP and NAD (Stitt *et al.*, 1989). The pellet was resuspended in 0.2 M KOH for starch quantitation. After a 30 min incubation at 100 °C, the pH was adjusted to 5.5 with 1 M acetic acid. The starch fraction was hydrolyzed with amylase (EC 3.2.1.1) and amyloglucosidase (EC 3.2.1.3), and assayed by enzymatic determination of released glucose (Stitt *et al.*, 1989).

Soluble proteins

Soluble proteins were assayed according to Bradford (1976) with chicken egg white albumin as a standard, after protein extraction from lyophilized tuber powder in 50 mM Tris-HCl, pH 7.5, containing 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF) (see Rivard *et al.*, 2006).

Minerals

Potassium, magnesium and copper were analyzed by emission spectrometry as described (AOAC, 2007f). The tuber samples were dried, precherred and ashed overnight in a muffle furnace at 550 °C. Ashed samples were treated with hydrochloric acid, taken to dryness, and dissolved in 5% (v/v) hydrochloric acid. The amount of each element was determined at the appropriate wavelength using inductively coupled plasma, by comparing emission of the unknown sample with the emission of standard solutions.

Amino acids

Amino acids were extracted in methanol : chloroform : H₂O 12 : 5 : 2, derivatized and analyzed by gas chromatography–MS according to Pearson and Nasholm (2001). Internal standards (Sigma-Aldrich) were used for quantification.

Glycoalkaloids

Total glycoalkaloids were extracted in acetic acid according to Sotelo and Serrano (2000), and quantified by HPLC using the Waters system controller 600-MS, a Waters 431 pump and the UV photodiode array detector 996 (Waters, Mississauga ON, Canada). A C-18 column was used for chromatographic separation, with a water : acetonitrile (50 : 50 + NH₄K₂HPO₄) mobile phase. The solvent flow rate and UV absorbance detector were adjusted at 1.0 mL/min and 280 nm, respectively.

Protein extraction for two-dimensional gel electrophoresis

Proteins for proteomic analyses were extracted according to Espen *et al.* (1999), with some modifications. In brief, 100 mg of lyophilized tuber powder was extracted in 750 µL of extraction buffer (50 mM Tris-HCl, pH 7.6, 0.25 M sucrose, 10 mM MgCl₂, 1 mM EDTA, 10 mM phenylmethylsulfonyl fluoride) for 30 min at 4 °C with gentle stirring, in the presence of 1% (w/v) polyvinylpyrrolidone. After centrifugation at 20 000 *g* for 30 min at 4 °C, the supernatant was recovered for protein precipitation in methanol/chloroform according to Wessel and Flügge (1984). In brief, 150 µL of supernatant was mixed with 600 µL of methanol and 150 µL of chloroform. After a 2-h incubation at -20 °C, 450 µL of double-distilled water was added, and the whole mixture was mixed by inversion. The samples were then centrifuged at 14 000 *g* for 30 min at 4 °C, and the pellets were washed with 600 µL of methanol before being recentrifuged. The precipitates were Speedvac-dried and solubilized in 0.5% IPG buffer (pH 3–10 or pH 4–7) (GE Healthcare) containing 8 M urea, 2% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1 propane-sulfonate (CHAPS) and 60 mM dithiothreitol. The mixtures were vortexed extensively and sonicated for 1 h. Protein content in the samples was determined according to Bradford (1976), with chicken egg white albumin as a protein standard.

Two-dimensional gel electrophoresis and image analysis

Two-dimensional gel electrophoresis first involved IEF (1st dimension), followed by standard 15% (w/v) sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) (2nd dimension). IEF was performed at 16 °C along a 4 to 7 (or 3 to 10) pH gradient in 13-cm Immobiline DryStrip gel strips (GE Healthcare), with 200 µg of leaf protein diluted in 250 µL of electrophoretic sample buffer (see above). Proteins were applied to the strips and resolved using an IPGphor apparatus (GE Healthcare). The program for IEF separation involved the following sequential steps: rehydration for 12 h at 30 V; for 1 h at 100 V; for 1 h at 500 V; for 1 h at 1000 V; for 1 h at 5000 V; and at 8000 V to reach 24 000 Vh. Following IEF, the strips were incubated for 25 min in a 50 mM Tris-HCl equilibration buffer, pH 8.8, containing 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 10 mg/mL dithiothreitol and traces of bromophenol blue, and used immediately for the second dimension. SDS-PAGE was performed in 1-mm thick polyacrylamide slab gels according to Laemmli (1970).

After migration, the gels were either fixed overnight in water containing 10% (v/v) acetic acid and 40% (v/v) methanol for subsequent protein staining; or electroblotted onto a nitrocellulose membrane without prior fixation and processed for immunodetection as described above. After fixation, the gels for staining were washed three times in water for 30 min, and the proteins were visualized by staining with the colloidal Coomassie blue Gel-Code reagent (Pierce, Rockford IL, USA). Gel images were digitalized and subjected to computer processing and image analysis, with three biological replicates for each plant line to allow for statistical assessments. Image analysis was carried out using the Phoretix 2D Expression software, v. 2005 (NonLinear USA). Spot

detection and background subtraction were performed following the supplier's recommendations. The gel containing the highest number of protein spots was identified, and used as a reference for protein spot matching. For each set of three gels (replicates), an average virtual gel was constructed that included protein spots found on at least two gels. Spot matching was performed with the average gels, and spots showing significantly variable intensities were identified based on a one-way ANOVA using an error rate of 5%.

Tryptic digests and protein identification

Protein spots for identification were cut out from the gels manually, washed for 15 min in double-distilled water, and incubated for 15 min in 250 µL of water/acetonitrile (1 : 1 v/v). After additional washes in acetonitrile and 100 mM NH₄HCO₃, the proteins were reduced with dithiothreitol, alkylated with iodoacetamide, and digested to peptides overnight at 37 °C with MS grade Trypsin Gold (Promega Corporation, Madison, WI, USA). The tryptic digests were sent to the Institute for Biomolecular Design (Edmonton, AB, Canada) or to the Génome Québec Innovation Centre at McGill University (Montréal, QC, Canada) for liquid chromatography-MS/MS with an electrospray ionization-ion trap mass spectrometer. Protein identification was performed using the MASCOT algorithm (<http://www.matrixscience.com>) with the following criteria for positive identification: a MASCOT score significant at $P < 0.05$, at least two matched peptides, and a minimal sequence coverage of 10%. The following search parameters were used for database mining: Type of search = MS/MS ion search; Fixed modifications = carbamidomethyl (Cys); Variable modifications = oxidation (Met); Mass values = monoisotopic; Peptide mass tolerance ± 1.5 Da; Fragment mass tolerance = ± 0.8 Da; Maximum missed cleavages = 1.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Table S1 Complement to table 4: LC-MS/MS identification of tuber proteins immunodetected with the anti-S/CDI antibodies.

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Table S1 Complement to Table 4: LC-MS/MS identification of tuber proteins immunodetected with the anti-S/CDI antibodies

Spot	Putative protein	Accession No. ^{a)}	Species	Mowse score	No. of peptides	Sequence cov. (%)	Peptide sequences
A	Proteinase inhibitor II	CAA27730	<i>Solanum tuberosum</i>	81	7	59	ACTLECGNLGFGICPR SEGSPENR GCNYYSANGAFICEGQSDPK ACPLNCDPHIAYSK SLIYPTGCTTCCTGYK GCYYFGK FVCEGESDEPK
B	Proteinase inhibitor II	CAA27730	<i>S. tuberosum</i>	97	7	60	ACTLECGNLGFGICPR SEGSPENR GCNYYSANGAFICEGQSDPK ACPLNCDPHIAYSK SLIYPTGCTTCCTGYK GCYYFGK FVCEGESDEPK
C	Proteinase inhibitor II	CAA27730	<i>S. tuberosum</i>	71	6	50	ACTLECGNLGFGICPR GCNYYSANGAFICEGESDPK NPNVCPR SLIYPTGCTTCCTGYK GCYYFGK FVCEGESDEPK
D	Proteinase inhibitor II	CAA27730	<i>S. tuberosum</i>	71	6	45	ACTLECGNLGFGICPR GCNYYSANGAFICEGESDPK NPNVCPR SLIYPTGCTTCCTGYK GCYYFGK FVCEGESDEPK
E	Serine proteinase inhibitor I (PSPI-21)	P58514	<i>S. tuberosum</i>	85	2	18	YNSDVGPSGTPVR LPSDATPVLDVTGK
F	PSPI-21	P58514	<i>S. tuberosum</i>	85	2	18	YNSDVGPSGTPVR LPSDATPVLDVTGK
G	Cathepsin D inhibitor	S10721	<i>S. tuberosum</i>	67	4	56	SPNSDAPCPDGVFR YNSDVGPSGTPVR VGNLNAYFR RCPEDGFCAK

Table S1 Contn'd

Spot	Putative protein	Accession No. ^{a)}	Species	Mowse score	No. of peptides	Sequence cov. (%)	Peptide sequences
H	Cathepsin D inhibitor	P58521	<i>S. tuberosum</i>	45	6	47	SPNSDAPCPDGVFR YNSDVGPSGTPVR FIPLSGGIFEDQLLNQFNIP TVK LCVSYTIWK VGNLNAYFR TMLLETGGTIGQADNSYFK
I	Aspartic proteinase inhibitor	CAA45723	<i>S. tuberosum</i>	179	4	20	SPNSDAPCANGIFR YNSDVGPSGTPVR LCVSYTIWK DNPLDVSFK
J	Aspartic proteinase inhibitor	AAB23206	<i>S. tuberosum</i>	534	12	43	ELNPDSSYR ELNPDSSYR GALGGDVYL GK YNSDVGPSGTPVR YNSDVGPSGTPVR LCVSYTIWK VGNLNAYFR VGNLNAYFR VGNLNAYFR TMLLETGGTIGQADSSYFK Oxidation (M) VGVIQNGK RLALVNENPLDVL FQEV
K	Aspartic proteinase inhibitor	P58518	<i>S. tuberosum</i>	60	6	60	SPNSDAPCPDGVFR YNSDVGPSGTPVR FIPLSTNIFEDQLLNQFNIP TVK VGNLNAYFR TMLLETGGTIGQADNSYFK LALVNENPLDVL FQEV
L	Proteinase inhibitor	BAA04152	<i>S. tuberosum</i>	141	2	12	SPNSDAPCANGVFR FNSDVGPSGTPVR
M	Proteinase inhibitor	BAA04152	<i>S. tuberosum</i>	65	8	51	ELDPR IISIGR GALGGDVYL GK FNSDVGPSGTPVR FIGSSSHFGPHIFEGELLNIQFDISTVK LCVSYTIWK VG DYDASLGTMLLETGGTIGQADSSWFK Oxidation (M) DNPLDVSFK

^{a)} Accession numbers are from the NCBI GenBank database (www.ncbi.nlm.nih.gov).