

Conversion of Exogenous Cholesterol into Glycoalkaloids in Potato Shoots, Using Two Methods for Sterol Solubilisation

Erik V. Petersson^{1*}, Nurun Nahar¹, Paul Dahlin², Anders Broberg³, Rikard Tröger⁴, Paresh C. Dutta⁵, Lisbeth Jonsson², Folke Sitbon¹

1 Department of Plant Biology and Forest Genetics, Uppsala BioCenter, Swedish University of Agricultural Sciences and Linnean Centre for Plant Biology, Uppsala, Sweden, **2** Department of Ecology, Environment and Plant Sciences, Stockholm University, Stockholm, Sweden, **3** Department of Chemistry, Uppsala BioCenter, Swedish University of Agricultural Sciences, Uppsala, Sweden, **4** Chemistry Division 1, Science Department, National Food Agency, Uppsala, Sweden, **5** Department of Food Science, Uppsala BioCenter, Swedish University of Agricultural Sciences, Uppsala, Sweden

Abstract

Steroidal glycoalkaloids (SGA) are toxic secondary metabolites naturally occurring in the potato, as well as in certain other Solanaceous plant species, such as tomato, eggplant and pepper. To investigate the steroidal origin of SGA biosynthesis, cut potato shoots were fed cholesterol labelled with deuterium (D) in the sterol ring structure (D₅- or D₆-labelled), or side chain (D₇-labelled), and analysed after three or five weeks. The labelled cholesterol and presence of D-labelled SGA were analysed by GC-MS and LC-MS/MS, respectively. When feeding D-labelled cholesterol solubilised in Tween-80, labelled cholesterol in free form became present in both leaves and stems, although the major part was recovered as steryl esters. Minor amounts of D-labelled SGA (α -solanine and α -chaconine) were identified in cholesterol-treated shoots, but not in blank controls, or in shoots fed D₆-27-hydroxycholesterol. Solubilising the labelled cholesterol in methyl- β -cyclodextrin instead of Tween-80 increased the levels of labelled SGA up to 100-fold, and about 1 mole% of the labelled cholesterol was recovered as labelled SGA in potato leaves. Both side chain and ring structure D labels were retained in SGA, showing that the entire cholesterol molecule is converted to SGA. However, feeding side chain D₇-labelled cholesterol resulted in D₅-labelled SGA, indicating that two hydrogen atoms were released during formation of the SGA nitrogen-containing ring system. Feeding with D₇-sitosterol did not produce any labelled SGA, indicating that cholesterol is a specific SGA precursor. In conclusion, we have demonstrated a superior performance of methyl- β -cyclodextrin for delivery of cholesterol in plant tissue feeding experiments, and given firm evidence for cholesterol as a specific sterol precursor of SGA in potato.

Citation: Petersson EV, Nahar N, Dahlin P, Broberg A, Tröger R, et al. (2013) Conversion of Exogenous Cholesterol into Glycoalkaloids in Potato Shoots, Using Two Methods for Sterol Solubilisation. PLoS ONE 8(12): e82955. doi:10.1371/journal.pone.0082955

Editor: Markus Grebe, Umeå Plant Science Centre, Sweden

Received: August 1, 2013; **Accepted:** October 29, 2013; **Published:** December 9, 2013

Copyright: © 2013 Petersson et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by The Swedish Research Council Formas Grant No. 229-2004-1576 and The Swedish Farmers' Foundation for Agricultural Research, Grant No. H0942171. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

* E-mail: erik.petersson@slv.se

Introduction

Sterols are major components of cellular membranes in eukaryotic organisms. In animals, cholesterol is the main sterol, whereas plants contain a mixture of sterols with varying length and saturation of the side chain [1]. For a long time, it was believed that plants did not contain cholesterol, but since the finding of cholesterol in potato and *Dioscorea spiculiflora* [2], cholesterol has been found in most plant species, although usually as a minor sterol. However, in some genera, e.g. the Solanaceae and Liliaceae, cholesterol constitutes a significant part of total sterols [3,4]. Besides having a role in membrane structure and function, it has been proposed that plant

cholesterol is a precursor of specific steroidal secondary metabolites, such as sapogenins and steroidal glycoalkaloids (SGA) [5]. The steroidal alkaloids are widely spread in *Solanum* and related genera, including important crop species such as the potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*) and eggplant (*Solanum melongena*). SGA are not restricted to these families, but are also found in e.g. the Asclepidaceae and Liliaceae. The SGA are toxic [6–9], and food authorities in many countries have established a maximum level of 200 mg total SGA/kg fresh weight (FW) in unpeeled potato tubers aimed for consumption [9,10].

The presence of SGA in potato plants has been known for almost a century [11], but the biosynthetic route has not yet

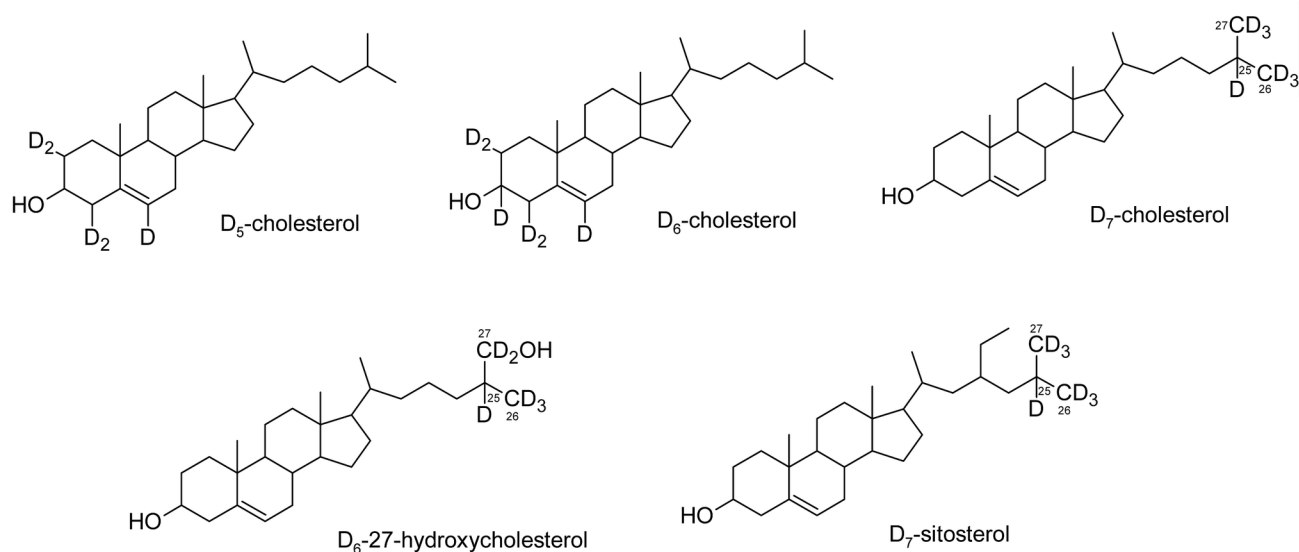


Figure 1. Chemical structures of deuterium (D) -labelled sterols used in the present study. Cut potato shoots were fed D₅- or D₆-cholesterol (labelled in the sterol ring structure), or D₇-cholesterol (labelled in the sterol side chain). Side chain-labelled D₆-27-hydroxycholesterol and D₇-sitosterol were used as controls.

doi: 10.1371/journal.pone.0082955.g001

been completely elucidated. According to the suggested biosynthetic pathway, cholesterol is a SGA precursor that via unknown mechanisms is converted to the aglycone solanidine, which is further glycosylated by sequential glycosylation reactions to the two major SGA in commercial potato, α -chaconine and α -solanine [5,7]. The latter steps of this pathway, i.e. the sequential glycosylations starting from solanidine, have been well characterised. Three enzymes and their respective genes have been isolated from potato, and their properties demonstrated *in vitro* and via transgenic plants [12-14]. Also in tomato, the genes responsible for glycosylation of the corresponding aglycone tomatidine have been identified [15,16].

With regard to the early steps of SGA biosynthesis and particularly the role of cholesterol as a precursor, the experimental evidence are however as of yet not fully convincing. Current models are to a large extent based on feeding studies with radioactive precursors in various solanaceous species. In potato, such studies include a report of the incorporation of radioactivity from cholesterol to solanidine [17]. Further, feeding experiments with cholesterol-4-[¹⁴C] to etiolated potato sprouts indicated that cholesterol was transformed into 26-hydroxycholesterol and cholest-4-en-3-one [18]. Other proposed conversions of cholesterol in Solanaceae have included a hydroxylation at C-26 and the direct replacement of the C-26 hydroxyl group by an amino group [19]. The latter mechanism has been challenged in two recent studies, indicating that the C-26 transamination is occurring via an aldehyde intermediate in tomato [16,20], potato and eggplant [20].

Indirect evidence for cholesterol as the precursor to SGA in potato has come from biochemical analyses of potato tubers,

where SGA biosynthesis was initiated after slicing tubers into discs [21]. A regulatory role in SGA biosynthesis was suggested for the sterol methyltransferase enzyme (SMT1), which catalyses the biosynthesis of sterols with an alkylated side-chain (C10-side chain). Inhibition of this enzyme leads to higher amounts of cholesterol (a C8-side chain sterol) [22]. Under conditions where this enzyme was up-regulated and the cholesterol biosynthesis inhibited in the tuber discs, no SGA biosynthesis occurred [23,24]. Further evidence came from transformation studies, where overexpression of the gene for SMT1 in potato resulted in lower amounts of cholesterol and of SGA [25].

The present study aimed at clarifying the role of cholesterol in the biosynthesis of SGA in potato plants. During the publication process, a similar study was published demonstrating side chain-labelled D₆- and D₇-cholesterol as SGA precursors in tomato and potato [20]. We here confirm and extend the results concerning the potato SGA metabolism. Using D₅-, D₆-, and D₇-cholesterol, as well as D₆-27-hydroxycholesterol and D₇-sitosterol (Figure 1) as substrates, we demonstrate that only cholesterol is incorporated into SGA, and that both the cholesterol ring structure and side chain are retained. Moreover, we show that solubilising deuterated cholesterol in methyl- β -cyclodextrin (MBD) results in a significantly stronger increase in the label incorporation into SGA, compared to the detergent Tween-80.

Results and Discussion

Uptake and distribution of D₅-labelled cholesterol in potato shoots

Potato shoots (cv. King Edward) were through the cut stem fed 200 µg D₅-cholesterol in a Tween-80/water suspension (Figure 2). At the end of the incubation period, the general appearance of the shoots was in most cases healthy, although a few shoots showed minor signs of leaf wilting. For mass spectrometry (MS) analysis of metabolites, the shoots were divided into upper and lower leaves and stems (Figure 2). The molecular ions and multiple reaction monitoring (MRM) transitions of both the endogenous and labelled metabolites are described in Table 1. The results showed that all D₅-cholesterol had been taken up and could be recovered from the shoots, and was distributed in both leaves and the stem (Table 2). A GC-MS chromatogram of labelled and endogenous cholesterol is shown in Figure S1. The main part, ca. 85 % of D₅-cholesterol, was recovered in the lower stem and in the lower leaves, and ca. 15% was present in the newly developed upper stem and leaves. About 80% of D₅-cholesterol was recovered after saponification of the sterol fraction, indicating that the major part was stored as steryl esters (Table 2).

Metabolism of D₅-labelled cholesterol in potato shoots

Potato shoots (cv. King Edward), fed as above, or with an equal amount of D₆-27-hydroxycholesterol (as a control), were analysed for SGA using LC-MS/MS. The *m/z* +5 MRM transitions for α-chaconine and α-solanine, showed in the D₅-cholesterol-treated materials that D₅-SGA was formed at low levels, but significantly higher than the ¹³C-derived background (Table 3). Similar results were obtained also in cv. Bintje (Table 3), thus confirming the conversion of cholesterol to SGA in another potato cultivar. Since the endogenous SGA was several orders of magnitude higher than the D₅-SGA, the D₅-SGA contents were corrected for the natural ¹³C-contribution to the +5 isotopic peaks. Based on the natural abundance of ¹³C, the natural content of the SGA *m/z* +5 MRM-transition from the molecular ion to the aglycone was estimated to be 0.0023 % in the blank samples. The evaluation of four control shoots in duplicate showed results quite near this value (α-chaconine 0.0035 % ± 0.0016 %; α-solanine 0.0037 % ± 0.0019 %). After ¹³C-correction, D₅-SGA levels ranged between 0.01–0.23 mg/kg FW, with the highest levels in lower leaves (Table 3). About 0.1 mole% of the added 200 µg D₅-cholesterol was recovered in D₅-SGA when summarized in leaves and stem. Approximately 85% of this D₅-SGA was located in the leaves (Table 2). By contrast, no increases of labelled SGA could be detected in shoots fed D₆-27-hydroxycholesterol despite monitoring several possible MRM transitions (not shown). In summary, the results demonstrate that the ring structure of D₅-cholesterol is incorporated into SGA, and indicate that 27-hydroxycholesterol is not used as precursor for SGA.

Levels of endogenous SGA and sterols in shoots fed D₅-labelled cholesterol

Addition of significant amounts of cholesterol might be anticipated to perturb the normal metabolism of sterols and

SGA. Hence, as a control, the effects of added D₅-cholesterol on SGA and sterol levels were studied in the different tissues.

Endogenous SGA levels in both cv. King Edward and cv. Bintje were generally highest in the upper, young leaves followed by the lower, older leaves (Table 3). For all the tissues investigated, the endogenous level of SGA, and the ratio between α-chaconine and α-solanine, was similar in control shoots and shoots fed D₅-cholesterol.

To study the effects of D₅-cholesterol feeding on sterol levels, both cholesterol and the other three major desmethylsterols in potato plants: sitosterol, campesterol and stigmasterol, were analysed in cv. King Edward (Table 4). Compared to blank controls, the shoots that had been fed D₅-cholesterol had similar levels of free cholesterol, and a similar ratio between endogenous cholesterol and sitosterol, stigmasterol, or campesterol. For obvious reasons, the shoots that had been fed D₅-cholesterol contained much higher cholesterol levels as compared to the control shoots. Earlier studies on mutants overproducing sterols have shown that plant cells are able to accommodate high amounts of sterols as steryl esters in lipid particles [26,27]. The main part of the added D₅-cholesterol was indeed recovered in esterified form, distributed similarly to its free form, i.e. the highest levels were found in the older parts of the plants, the lower stem and the lower leaves (Table 4). The ratios between esterified and free cholesterol in the different tissues were similar for the D-labelled and the endogenous species. Still, when considering only the free cholesterol, the shoots fed D₅-cholesterol had in all tissues higher amounts of total cholesterol (endogenous and D₅-cholesterol) than the control plants. This raises the question of how the shoots managed to maintain membrane function. Since the general appearance of the treated shoots was not different from controls, we suggest that the sterol composition in the membranes was kept stable and that the added D₅-cholesterol became localised in a different subcellular pool compared to the endogenous sterols. It has recently been shown in tobacco and Chinese cabbage, that cholesterol is the major sterol in the phloem exudate [28]. It is also of interest that one of the enzymes in sterol biosynthesis, Δ⁷-sterol-C₅-desaturase, was found localised to lipid droplets which were clearly seen in vascular tissue [29]. It is tempting to speculate that the additional amounts of cholesterol in the D₅-cholesterol-fed shoots occur in a transport form, possibly in the phloem sap. Nevertheless, the results demonstrate that the sterol feeding process is not associated with an alteration of the endogenous metabolism of sterols or SGA.

Increased conversion of cholesterol to SGA by solubilisation in methyl-β-cyclodextrin

The results from feeding D₅-cholesterol solubilised in Tween-80 suggested that the D₅-cholesterol was not available for conversion into SGA to the same extent as endogenous cholesterol. In order to investigate this aspect, we carried out a feeding experiment in cv. King Edward comparing cholesterol solubilised in Tween-80 or in methyl-β-cyclodextrin (MBD), another substance commonly used for sterol solubilisation. In this experiment, both D₆(ring)- and D₇(side chain)-labelled cholesterol were used for two main reasons. The first reason is

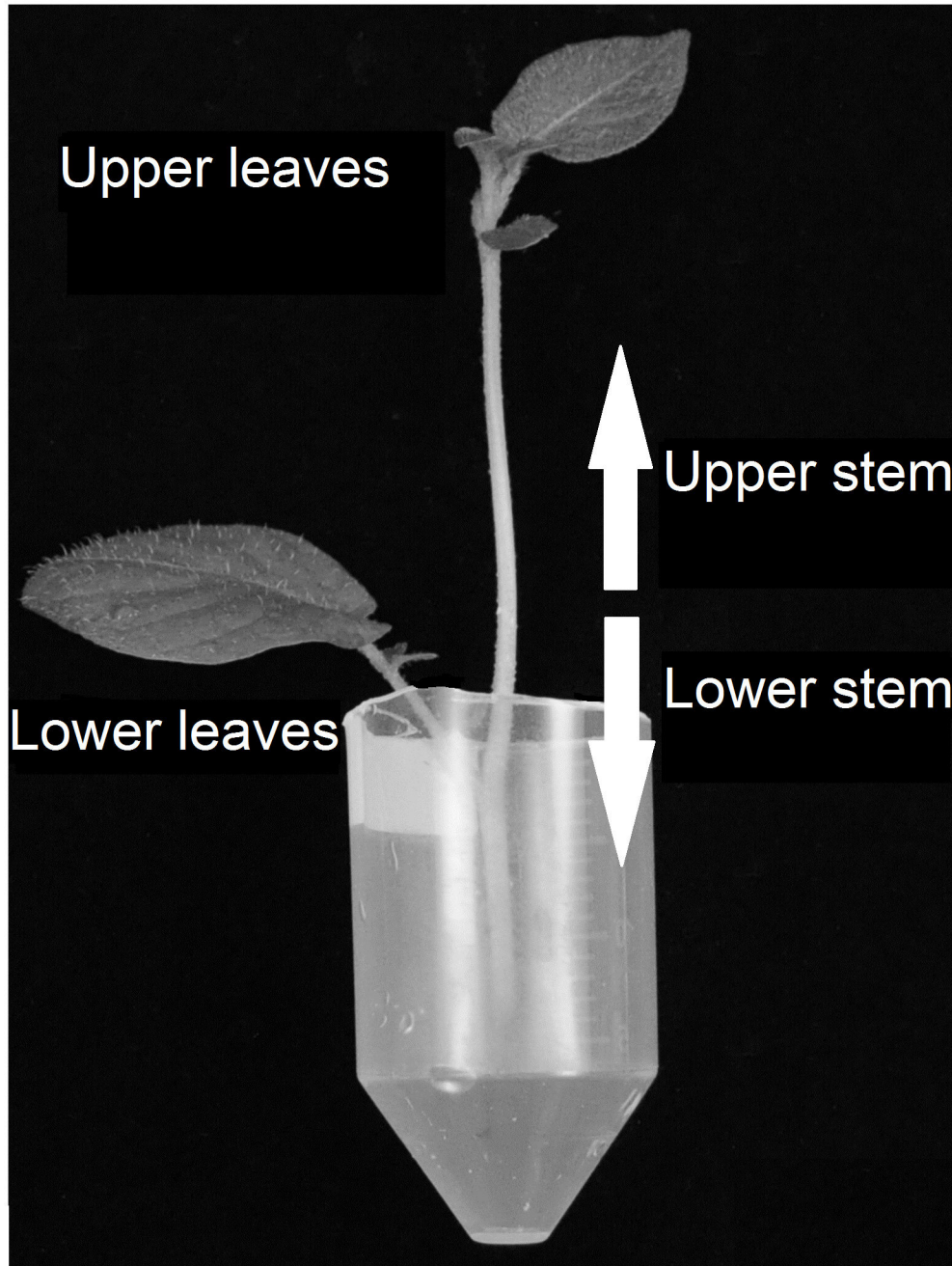


Figure 2. Experimental set-up for sterol feeding studies. Young potato shoots were trimmed to a final length of 10 cm, fed labelled sterols through the cut stem, and incubated for either three or five weeks. After the feeding period, shoots were divided into four parts consisting of upper leaves, lower leaves, upper stem, and lower stem. Materials from up to three separate shoots were pooled when necessary.

doi: 10.1371/journal.pone.0082955.g002

that the natural ^{13}C -contribution to the formed D-SGA is lower for heavier substrates, such as D_6 -cholesterol, and the second reason is that the side chain-labelled cholesterol gives additional information about the fate of the side chain (Figure 1). Water blanks were used as controls. As an additional control, feeding was also carried out with D_7 -labelled sitosterol,

to investigate whether conversion to SGA is specific for cholesterol. All samples were monitored for endogenous as well as m/z +5, +6, and +7 MRM-transitions (Table 1).

Endogenous and D-labelled SGA were analysed in the upper and lower leaves after 3 and 5 weeks (Table S1). A representative LC-MS/MS chromatogram is shown in Figure

Table 1. Molecular ions and fragments monitored for endogenous and deuterium (D) -labelled cholesterol and SGA.

Compound	Molecular ion [M] ⁺	LC-MS/MS: MRM transition to	
		unique fragment ion (m/z)	
Cholesterol	386		
D ₅ -cholesterol (R)	391		
D ₆ -cholesterol (R)	392		
D ₇ -cholesterol (SC)	393		
Sitosterol	414		
D ₇ -sitosterol (SC)	421		
α-solanine	852	398; 98	
α-chaconine	868	398; 98	
D ₅ -α-chaconine (R)	857	403; 98	
D ₅ -α-solanine (R)	873	403; 98	
D ₆ -α-chaconine (R)	858	404; 98	
D ₆ -α-solanine (R)	874	404; 98	
D ₅ -α-chaconine (SC) [*]	857	403; 103	
D ₅ -α-solanine (SC) [*]	873	403; 103	

Sterols were analysed by GC-MS and SGAs were analysed by LC-MS/MS. Peak identities were verified by comparison to authentic standards. The D labels were either in the ring structure (R), or in the side chain (SC), of the sterols and SGA. Presence (or absence) of side chain label was confirmed in SGA by Multiple Reaction Monitoring (MRM) of mass transitions from the molecular ion to a SC-specific part of the aglycone (e.g. m/z 103 or 98 for D₅-label or no label, respectively). The full aglycone fragment was monitored at m/z 398, 403 and 404 (endogenous and labelled).

^{*} formed from D₇-cholesterol (SC)

doi: 10.1371/journal.pone.0082955.t001

Table 2. Recovery of D₅-cholesterol fed to potato shoots.

	D ₅ -cholesterol released from					
	D ₅ -cholesterol		D ₅ -cholesterol released from steryl esters		D ₅ -SGA	
	µg	% of total	µg	% of total	µg	% of total
Upper stem	5,1	12	17,5	11	0,05	11
Lower stem	31,2	71	94,9	59	0,02	4
Upper leaves	0,7	2	9,3	6	0,03	7
Lower leaves	6,6	15	39,3	24	0,35	78
Total	43,6	100	161	100	0,45	100

Cut potato shoots (cv. King Edward) were fed 200 µg D₅-cholesterol solubilised in Tween-80, and sterol and SGA metabolites were analysed after three weeks by GC-MS and LC-MS/MS, respectively. Cholesterol and SGA: n=2 samples per data point (biological duplicate), steryl esters: n=1 sample per data point.

doi: 10.1371/journal.pone.0082955.t002

S2. When comparing all samples, significantly more D-SGA was formed with MBD (p< 0.001; two-tailed, paired Student's *t*-test) as shown in Figure 3A. MBD samples had an average amount of D-SGA formed of 5.12 µg/shoot; about 40 times higher than the average in Tween-80 samples (0.13 µg/shoot). For individual samples, the levels of labelled SGA in shoots fed D₆-cholesterol or D₇-cholesterol increased up to 100-fold when

Table 3. Endogenous and D₅-SGA levels in potato shoots fed D₅-cholesterol.

	Endogenous SGA (mg/kg FW)			D ₅ -SGA (mg/kg FW)		
	Total SGA	α-solanine	α-chaconine	Total SGA	α-solanine	α-chaconine
cv. 'King Edward'						
Controls (Blanks)						
Upper stem	180 (±81)	37 (±18)	143 (±63)	ND	ND	ND
Lower stem	101(±66)	26 (±16)	75 (±50)	ND	ND	ND
Upper leaves	1447(±534)	367 (±152)	1080 (±382)	ND	ND	ND
Lower leaves	546 (±318)	163 (±106)	383 (±212)	ND	ND	ND
cv. 'King Edward' fed D₅-cholesterol						
Upper stem	76 (±3)	15 (±1)	61 (±2)	0.02 (±0.00)	0.00	0.02 (±0.00)
Lower stem	72 (±3)	22 (±1)	50 (±2)	0.02 (±0.00)	0.00	0.02 (±0.00)
Upper leaves	1539 (±311)	378 (±73)	1161 (±238)	0.05 (±0.01)	0.01 (±0.00)	0.04 (±0.01)
Lower leaves	308 (±100)	77 (±25)	231 (±75)	0.23 (±0.02)	0.03 (±0.01)	0.20 (±0.02)
cv. 'Bintje'						
Controls (Blanks)						
Upper stem	106	39	67	ND	ND	ND
Lower stem	63	22	41	ND	ND	ND
Upper leaves	467	139	328	ND	ND	ND
Lower leaves	67	17	50	ND	ND	ND
cv. 'Bintje' fed D₅-cholesterol						
Upper stem	194	71	124	0.01	0.00	0.01
Lower stem	256	106	150	0.02	0.00	0.02
Upper leaves	377	98	279	0.13	0.01	0.12
Lower leaves	188	39	149	0.15	0.02	0.13

Cut potato shoots (cvs. King Edward and Bintje) were fed 200 µg D₅-cholesterol solubilised in Tween-80, and SGAs were analysed after three weeks by LC-MS/MS. Mean value ± range of measurements in biological duplicates (n=2 samples) for cv. King Edward, and a single analysis for cv. Bintje (n=1 sample). ND = not detected.

doi: 10.1371/journal.pone.0082955.t003

cholesterol was administered in MBD, as compared to Tween-80 (Figure 3B-E).

Table 4. Sterol levels in potato shoots fed D₅-cholesterol.

	D ₅ -									
	Sitosterol		Campesterol		Stigmasterol		Cholesterol		cholesterol	
	(mg/kg FW)	(mg/kg FW)	(mg/kg FW)	(mg/kg FW)	(mg/kg FW)	(mg/kg FW)	(mg/kg FW)	(mg/kg FW)	(mg/kg FW)	(mg/kg FW)
	FS	SE	FS	SE	FS	SE	FS	SE	FS	SE
Controls (Blanks)										
Upper stem	8.8 (±5.2)	69	2.6 (±1.5)	20	0.6 (±0.3)	3.8	1.2 (±0.2)	6.7	ND	ND
Lower stem	6.0 (±1.9)	18	1.3 (±0.6)	3.3	0.4 (±0.0)	1.1	0.5 (±0.1)	1.2	ND	ND
Upper leaves	12.0 (±6.9)	70	5.6 (±3.5)	31	0.7 (±0.3)	4.6	3.0 (±1.9)	14	ND	ND
Lower leaves	14.0 (±3.1)	76	9.0 (±1.1)	30	1.0 (±0.3)	3.4	2.8 (±0.2)	10	ND	ND
Fed D₅-cholesterol										
Upper stem	6.5 (±3.1)	38	2.7 (±1.8)	14	0.4 (±0.1)	1.3	1.3 (±0.3)	4.3	4.5 (±4.1)	17
Lower stem	9.3 (±0.6)	36	2.7 (±0.9)	9.8	0.6 (±0.0)	4.6	1.0 (±0.1)	4.9	42 (±31)	126
Upper leaves	12.0 (±0.5)	123	7.3 (±0.3)	37	0.7 (±0.2)	9.1	3.7 (±0.0)	35	2.1 (±0.7)	29
Lower leaves	11.0 (±4.1)	88	7.5 (±2.3)	25	0.5 (±0.1)	4.6	3.6 (±0.5)	20	9.3 (±0.7)	51

Cut potato shoots (cv. King Edward) were fed 200 µg D₅-cholesterol solubilised in Tween-80, and desmethylsterol levels were analysed after three weeks using GC-MS. Mean value ± range of measurements in biological duplicates (n=2 samples), or single analysis for conjugated sterols (n=1 sample). FS, free sterol; SE, sterol esters; ND, not detected.

doi: 10.1371/journal.pone.0082955.t004

For Tween-80 samples, the shoots fed D-labelled cholesterol during 5 weeks had slightly higher levels D-SGA than the shoots fed during 3 weeks. This difference was not observed in MBD samples. For all shoots, the levels of D-labelled SGA tended to decrease with time in the upper leaves and increase in the lower leaves. Slightly higher amounts of D-labelled SGA were formed from D₆-cholesterol compared to D₇-cholesterol (Table 5). When D-labelled cholesterol was administered in MBD, the absolute amounts of labelled SGA equalled or were higher than those of labelled free cholesterol (Table 5). In the leaves of plants fed D₆-cholesterol or D₇-cholesterol, ca. 1-3 mole% of the added 200 µg D-labelled cholesterol was recovered in free cholesterol, and typically 1 mole% in SGA.

In shoots fed D₇-sitosterol, the recovery of D₇-sitosterol in the leaf tissue was somewhat lower than the corresponding recovery in shoots fed D₆- and D₇-cholesterol (Table 5), possibly reflecting a lower solubility or transport rate for sitosterol in MBD as compared to cholesterol. No SGA of any D-label was detected in these shoots, indicating that sitosterol is not converted into SGA (Figure 3B-E, Table 5, Table S1).

As expected, it was found that the ring-labelled D₆-cholesterol formed D₆-SGA. However, the side-chain labelled D₇-cholesterol formed D₅-SGA (*m/z* +5), both regarding α-solanine and α-chaconine, rather than the expected D₇-SGA

(*m/z* +7). This means that two hydrogen atoms from the side chain are detached during the closure of the nitrogen-containing ring system, and confirms recent results showing loss of two hydrogen atoms from the C-26 position during ring closure [20]. Moreover, our results show that the hydrogen loss is specific to the cholesterol side chain, and do not occur from the A+B rings. The conversion of different cholesterol substrates to their presumed end products is outlined in Figure 4.

One factor worth considering is that levels of endogenous SGA are several orders of magnitude higher than the formed D₅- and D₆-SGA. The natural ¹³C-contribution to the *m/z* +6 MRM is only 0.0001%, and thereby negligible. On the other hand, for the formed D₅-SGA, the ¹³C-contribution to the *m/z* +5 MRM is 0.0023% and could make a significant contribution to this peak. However, shoots fed D₇-cholesterol in MBD formed D₅-SGA well above what is significantly affected by ¹³C-contribution. This contrasts to some extent to the incorporation of D₅-cholesterol using Tween-80 (Table 3), where lower levels of D₅-SGA were formed, and thus required correction.

Analysis of hydroxysterol intermediates

One of the suggested intermediates in the biosynthesis of SGA from cholesterol is 26-hydroxycholesterol [18,20]. Compared to what is known about the metabolism of plant sterols, considerably less is known about that of hydroxysterols. However, recent studies have clearly demonstrated hydroxysterols as endogenous sterol metabolites in plants [16,30]. Using GC-MS and authentic hydroxysterol standards, we identified 26-hydroxycholesterol (Figure S3) as well as 22-hydroxycholesterol as endogenous hydroxysterols in leaves of potato, but not in Arabidopsis, a species lacking SGA (Table S2). Thus, formation of 26-hydroxycholesterol may be an initial step in potato SGA biosynthesis. However, an attempt to detect D-labelled 26-hydroxycholesterol was negative, although this may be explained by the low endogenous 26-hydroxycholesterol levels (0.18 mg/kg FW). Based on the proportions between the endogenous intermediate and end product, it seems reasonable to assume that substantial amounts of fed leaf material will be required for analysis of labelled intermediates. More research is needed to optimise hydroxysterol labelling conditions *in vivo*, and the analytical methods, to be able to investigate whether the isotopic enrichment in the pool of specific hydroxysterols is consistent with a precursor role in SGA biosynthesis.

Given the structure required to close the N-containing ring system in solanidine (fused cyclic amine structure) compared to that in tomatidine (oxo-aza spiro structure), we speculate that 22-hydroxycholesterol is an endogenous hydroxysterol in potato, but not an important intermediate in the biosynthesis of potato SGA. The suggested 22,26-dihydroxycholesterol intermediate found in tomato [16] could not be investigated due to lack of adequate standards. We presume that also this compound is more relevant in tomatine biosynthesis. Based on these considerations, a model for biosynthesis of potato SGA from cholesterol is shown in Figure S4.

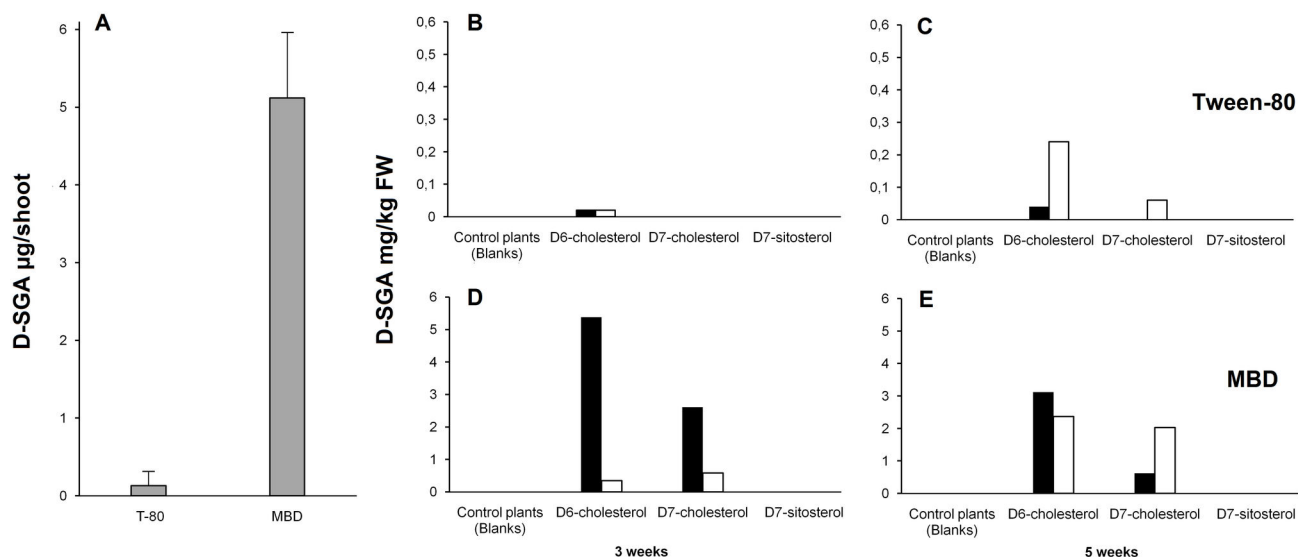


Figure 3. Formation of deuterium (D)-labelled SGA in potato shoots after feeding with D-labelled sterols solubilised in Tween-80 or methyl-β-cyclodextrin (MBD). Cut potato shoots (cv. King Edward) were fed 200 µg D₆-cholesterol, D₇-cholesterol, or D₇-sitosterol solubilised in Tween-80 or MBD, and incubated during either three or five weeks. After the incubation period, the levels of D-SGA (α-solanine and α-chaconine) were analysed in upper leaves (black bars) and lower leaves (white bars) using LC-MS/MS. (A) Graphical illustration of the difference between Tween-80 (T-80) and MBD feedings. Mean value ± SD for the amount of D-SGA formed in leaves up to 5 weeks. A difference between the treatments (n=4 full plants each) was significant at p<0.001 (paired Student's *t*-test). (B) to (E) individual analyses, each representing a single analysis of materials from 2-3 pooled plants: (B) Tween-80, 3 weeks; (C) Tween-80, 5 weeks; (D) MBD, 3 weeks; (E) MBD, 5 weeks. Note the different scales for Tween-80 and MBD. No D-SGA was formed in parallel analyses of blank controls, or shoots fed D₇-sitosterol.

doi: 10.1371/journal.pone.0082955.g003

Choice of solubilisation method

Collectively, our results demonstrate a specific conversion of D-labelled cholesterol into D-labelled SGA, with retention of both the cholesterol side chain and ring structure labels. Moreover, solubilising cholesterol in MBD instead of Tween-80, renders the sterol more available for conversion into SGA. Tween-80 is a nonionic surfactant (i.e. without ionic interaction) and emulsifier, consisting of a hydrophilic and a lipophilic group. As a surface-active agent, it has the potential to form a micellar structure at or above a concentration threshold known as the critical micelle concentration in solution. In micelles, the hydrophobic parts of a surfactant molecule associate to form a core region, whereas the hydrophilic parts position between the core and the external aqueous medium. In this way, the hydrophobic core gets stabilised by the hydrophilic shell [31]. Cyclodextrins, on the other hand, are cyclic oligosaccharides composed of a hydrophilic outer surface and a lipophilic central cavity that have the capacity to bind lipophilic compounds in their hydrophobic part [32]. The water-soluble MBD can form soluble inclusion complexes with cholesterol, thereby enhancing its solubility in aqueous solution. Our results show that forming an inclusion complex of cholesterol with MBD results in a much higher conversion rate of D-labelled cholesterol to SGA. This may be due to a more efficient uptake and transport in

Table 5. Deuterated (D) SGA and sterols in potato shoots fed D-labelled cholesterol or sitosterol solubilised in methyl-β-cyclodextrin (MBD).

Sterol fed	Endogenous sterol		Deuterium-labelled sterol		Deuterium-labelled SGA	
	3 weeks	5 weeks	3 weeks	5 weeks	3 weeks	5 weeks
	µg/shoot	µg/shoot	µg/shoot	µg/shoot	µg/shoot	µg/shoot
D ₆ -cholesterol	8.7	8.5	3.4	3.0	5.7	6.0
D ₇ -cholesterol	7.6	22.4	1.5	6.2	4.5	4.3
D ₇ -sitosterol	37.2	22.6	0.8	1.1	ND	ND

Cut potato shoots (cv. King Edward) were fed 200 µg D-labelled cholesterol or sitosterol solubilised in MBD, grown for either three or five weeks, after which sterols and SGA were analysed by GC-MS and LC-MS/MS, respectively. The amount per shoot is calculated from mean values of a single analysis of upper and lower leaves, respectively, that were pooled from 2-3 independent plants. ND, not detected.

doi: 10.1371/journal.pone.0082955.t005

combination with a more suitable availability for enzymes in the following steps in the conversion to SGA.

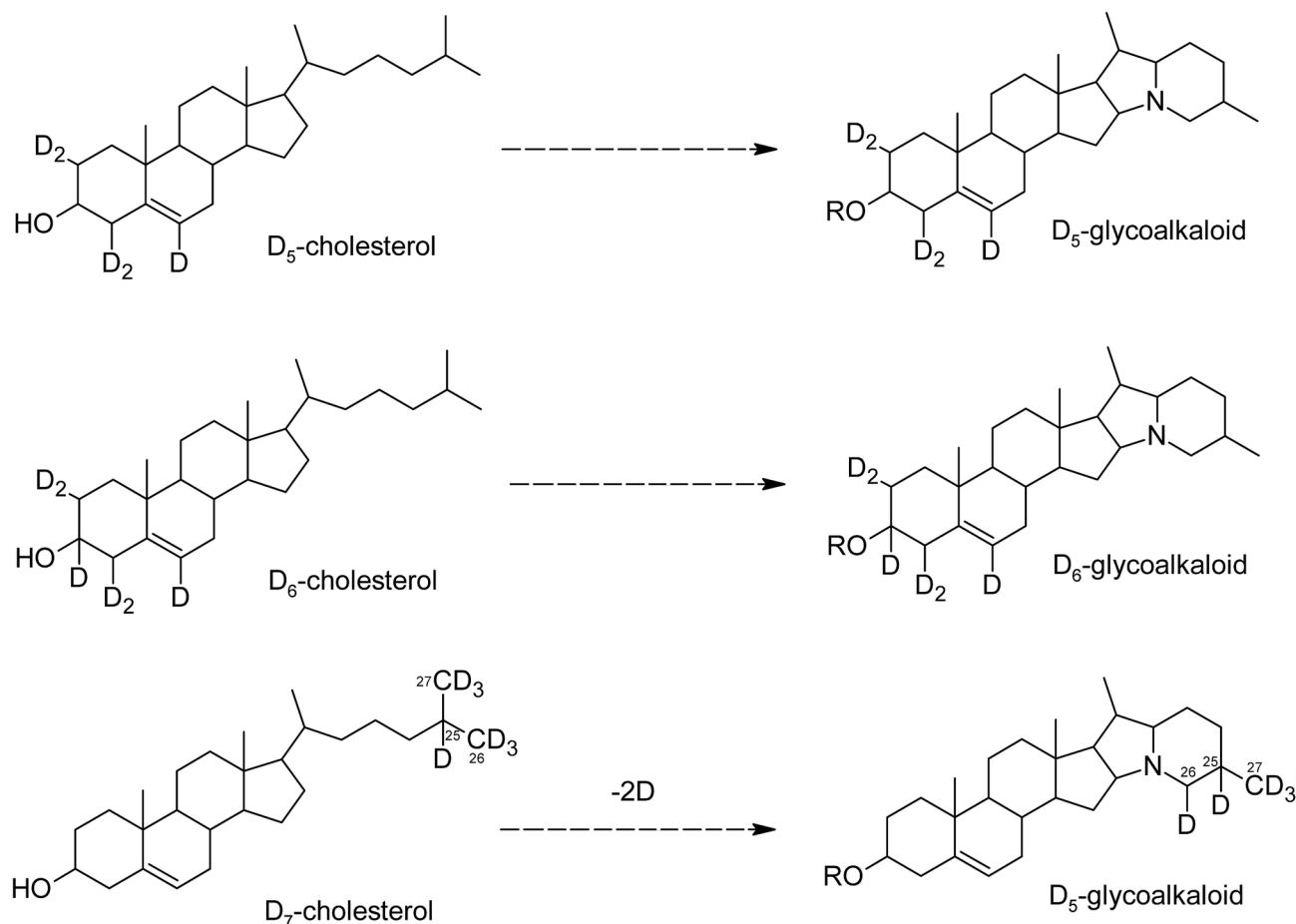


Figure 4. A model for the conversion of different deuterium (D) -labelled cholesterol substrates to D-labelled glycoalkaloids. Cut potato shoots were fed D_5 - or D_6 -cholesterol (labelled in the sterol ring structure), or D_7 -cholesterol (labelled in the sterol side chain). All these labels were converted *in vivo* to D-labelled SGA. Labels in the ring structure were intact after conversion to SGA, but two D atoms were lost from the side-chain label, indicative of an aldehyde formation, likely at the C-26 position. R signifies either OH (solanidine), chacotriose (α -chaconine), or solatriose (α -solanine).

doi: 10.1371/journal.pone.0082955.g004

Conclusions

An initial challenge was to solubilise a lipophilic compound, cholesterol, in a solution that would facilitate uptake and metabolism in plant tissues. We demonstrate here that the choice of solubilisation method has a profound impact on the conversion of cholesterol into SGA in cut potato shoots. When forming an inclusion complex of cholesterol with MBD, cholesterol is up to 100-fold more efficiently converted to SGA, than when forming micelles with Tween-80. In the leaves of plants fed D_6 -cholesterol or D_7 -cholesterol solubilised with MBD, ca. 1-3 mole% of the added D-labelled cholesterol (200 μ g) was recovered in free cholesterol, and typically 1 mole% in SGA. Feeding side chain D_7 -labelled cholesterol resulted in D_5 -labelled SGA, indicating that two hydrogen atoms are released from the side chain during closure of the nitrogen-containing ring system. Feeding with D_7 -sitosterol or D_6 -27-hydroxycholesterol did not produce detectable amounts of

labelled SGA. In conclusion, we have demonstrated a superior performance of MBD for the delivery of cholesterol in plant tissue feeding experiments, and given firm evidence for cholesterol as a specific precursor of SGA in potato. Our results confirm and extend previous studies that cholesterol constitutes an SGA precursor in potato plants [5,20], and can now be used as a base for further metabolic studies of SGA as well as other sterol-derived secondary metabolites in potato and other plant species.

Materials and Methods

Plant materials, and feeding conditions

Potato tubers of cvs. 'King Edward' and 'Bintje' were stored in the dark for two weeks at 20 °C to induce sprouting. The tubers were grown on soil in a growth chamber kept at 22 °C, and received white fluorescent light at a fluence rate of 110

$\mu\text{moles m}^{-2} \text{ s}^{-1}$ during a 16 h photoperiod. Shoots 15–20 cm long were harvested and trimmed to 10 cm by cutting off the base stem. The shoots were then weighed and placed into plastic tubes with tap water for three days to recover from any wounding stress (Figure 2). Solutions with D_5 -cholesterol (A, B-ring labelled; 2,2,4,4,6- D_5 ; Medical Isotopes Inc, Pelham, NH, USA) and D_6 -27-hydroxycholesterol (side-chain labelled; 25,26,26,26,27,27- D_6 , Avanti Polar Lipids Inc., Alabaster, AL, USA) were prepared by dissolving the sterols in 99.5% ethanol containing 5 mg Tween-80 (Riedel-de Haën, Hannover, Germany) and drying under nitrogen gas. The solids were then re-dissolved in 100 μl water containing 2% ethanol, and diluted to 1 ml with water. Tubes with 200 μg solubilised sterol was used for the labelling experiment, while the tubes for the control plants (blanks) were filled with a commercial fertiliser solution (Blomstra, Cederöth AB, Upplands Väsby, Sweden). All plants were grown in a growth chamber under the same growth condition as mentioned above. When the sterol solution (or fertiliser solution) after 3 to 5 days had been taken up, the tubes were refilled with additional fertiliser solution until three weeks in total had passed.

To form complexes with methyl- β -cyclodextrin (MBD), labelled sterols were dissolved following the protocol of Klein et al. [33], slightly modified by adding 400 μl isopropanol instead of chloroform. Exactly 200 μg of dissolved sterols, corresponding to 74 μl of the labelled sterol-MBD mixture, was diluted with autoclaved water to a final volume of 1 ml, which was fed to the cut shoots. D_6 -cholesterol (A,B-ring labelled; 2,2,3,4,4,6- D_6 ; Cambridge Isotopes, Andover, MA, USA), D_7 -cholesterol (sidechain-labelled; 25,26,26,26,27,27- D_7 ; Medical Isotopes Inc, Pelham, NH, USA), or D_7 -sitosterol (side chain-labelled; 25,26,26,26,27,27- D_7 ; Medical Isotopes Inc, Pelham, NH, USA) were added to shoots prepared as above. Blank control plants were fed only tap water. In parallel, the same amount of D_6 -cholesterol, D_7 -cholesterol and D_7 -sitosterol was dissolved in Tween-80 as described earlier and fed to separate shoots. After uptake of the sterol solution, tap water was added during seven days to prevent any interaction between nutrients and the sterol-MBD complex. The following weeks, fertiliser solution suspended in tap water (1:1000) was added to the shoots. Blank controls were treated the same way.

At the end of the feeding experiments (i.e. after 3 or 5 weeks), shoots were quickly rinsed with tap water, and divided into four parts consisting of upper and lower leaves; as well as upper and lower stems (Figure 2), and frozen in liquid nitrogen. When necessary, pools of up to three separate shoots were made to obtain sufficient materials for analysis.

Sterol extraction and subsequent analysis with GC-MS

Free 4-desmethylsterols were analysed in leaf and stem samples of 'King Edward' shoots. In brief, samples were homogenised in a mortar using liquid nitrogen, transferred to a glass tube, followed by addition of 43 μg desmosterol (Sigma-Aldrich, Schnellendorf, Germany) as internal standard (IS). Total lipids were extracted with 5 ml chloroform:methanol (2:1, v/v) for 1 h at 70 °C. When sterols released from conjugated forms were measured, an additional saponification step was applied

[25]. The sterol-containing chloroform phase was removed, dried under nitrogen gas, and reconstituted in 3 ml hexane. The extracts were purified with a solid phase extraction using ISOLUTE® Si 500 mg/6 ml cartridges (Biotage, Uppsala, Sweden) and the following procedure: activation with 3 ml hexane, loading the sample (in 3 ml hexane), washing with 9 ml hexane, eluting with 3 ml hexane:ethyl acetate (50:50 v/v). The eluate was dried under nitrogen gas and reconstituted in 1 ml hexane. The 4-desmethylsterols were analysed by GC-MS and quantified relative to the IS. Standards used for sterol identification were: endogenous cholesterol (Sigma-Aldrich, Schnellendorf, Germany); 2,2,4,4,6- D_5 -cholesterol, 25,26,26,26,27,27,27- D_7 -cholesterol, 25,26,26,26,27,27,27- D_7 -sitosterol (all from Medical Isotopes Inc, Pelham, NH, USA); 2,2,3,4,4,6- D_6 -cholesterol (Cambridge Isotopes, Andover, MA, USA); as well as the other major 4-desmethylsterols.

Sterols were characterised and identified by GC-MS. Free sterol analysis was performed on a GC8000 Top Series gas chromatograph (CE Instruments, Milan; Italy) with a DB35-MS column (15 m x 0.20 mm, 0.33 μm film thickness) from J&W Scientific (J&W Scientific, Folsom, CA, USA) coupled to a Voyager mass spectrometer (Finnigan, Manchester, UK). Injector temperature was 260 °C, and oven conditions were 60 °C for 1 min and raised to 270 °C at rate of 30 °C/min and maintained for 2 min and then raised to 290 °C at a rate of 1 °C/min. The temperatures of the transfer line to the MS and the detector were set to 290 °C. Helium was used as carrier gas at a constant pressure of 80 kPa. The samples were injected (1 μl) in split mode (ca. 50:1). Full scan mass spectra (m/z 50–450) were recorded at 70 eV. Both endogenous and labelled sterols were quantified by their total ion count (TIC) against the IS.

Hydroxycholesterols were extracted and purified using 19-hydroxycholesterol as an internal standard according to Beste et al. [30], with some modifications. The starting size of the sample was 20 grams of leaves, both upper and lower, in order to get enough material for quantification. The sample was extracted and concentrated throughout the procedure to a final volume of 400 μl TMSi derivative dissolved in hexane, which was analysed using GC-MS on a HP5890 GC (Hewlett-Packard, Palo Alto, CA, USA) equipped with an HP-5MS column (30 m x 0.25 mm, 0.25 μm film thickness) connected to a HP5970 MSD. Injector temperature was 260 °C, and oven conditions were 60 °C for 1 min and raised to 290 °C at rate of 50 °C/min and maintained for 10 min and then raised to 300 °C at rate of 1 °C/min and maintained for 15 min. The temperatures of the transfer line to the MS and the detector were set to 300 °C. Helium was used as carrier gas at a constant flow of 1 ml/min. The samples were injected (1 μl) in splitless mode. Hydroxysterols were analysed (at 70 eV) in selected ion monitoring (SIM) mode for increased sensitivity, and quantified against the internal standard using the major ion fragments for the hydroxycholesterol standards (m/z 546; 456; 417; 366; 353; 173; 145; 131; 129; 83; 73). Corresponding ion fragments for labelled counterparts were also monitored in samples fed labelled cholesterol. The standards used for comparisons were: 19-hydroxycholesterol, 20 α -hydroxycholesterol, 22(R)-hydroxycholesterol (Research Plus

Inc., Barnegat, NJ, USA), and 26-hydroxycholesterol (Avanti Polar Lipids Inc., Alabaster, AL, USA).

Glycoalkaloid extraction and analysis with LC-MS/MS and LC-UV

SGA were extracted from leaves and stems and purified according to Petersson et al. [34]. Solamargine was during the initial extraction step added as an internal standard, corresponding to 100 µg/g (FW) potato tissue. A mass ratio of 1 part sample and 4 parts of extraction liquid was used. The LC-MS/MS equipment used was an Agilent Technologies 6410 Triple Quadrupole with an Agilent 1200 Series HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a Synergi Fusion-RP column (4 µm, 2.1 mm x 75 mm) from Phenomenex (Torrance, CA, USA). The mobile phase was 22 % acetonitrile acidified with 0.1 % formic acid, and the flow rate was 0.5 ml/min. The mass spectrometer was used with a source voltage of 4 kV. MRM transitions monitored are shown in Table 1, and the collision energies used to fragment the molecular ions of α-solanine and α-chaconine (both endogenous and labelled) were 85 eV (to fragments *m/z* 398; 403; 404) and 95 eV (to fragments *m/z* 98; 103).

Endogenous SGA levels were quantified using LC-UV rather than LC-MS/MS, since the UV response was linear over a greater range of concentrations, and endogenous SGA levels were high enough for this less sensitive technique. After quantification with LC-UV, the levels of the labelled SGA were calculated by their relative LC-MS/MS response within the same sample, which proved to be constant and unaffected by ion suppression. The LC-UV equipment was an HP 1100 series (Hewlett Packard, Waldbronn, Germany) with a G1312A binary pump and a G1314A UV-detector set to 202 nm. Separation was achieved with a Hypersil Gold C18 column (5 µm, 2.1 mm x 150 mm) from Thermo Scientific (Waltham, MA, USA) using a mobile phase consisting of 10 mM phosphate buffer pH 7.6 with 36 % acetonitrile, at a flow rate of 0.5 ml/min. The standards used for the analyses were α-chaconine 95% (Sigma-Aldrich, Schnellendorf, Germany), α-solanine 95% (MP Biomedicals, Solon, OH, USA) and solamargine >98 % (Glycomix, Reading, UK).

Supporting Information

Figure S1. GC-MS chromatogram from an analysis of endogenous- and deuterium (D) -labelled cholesterol in potato leaves. Cut potato shoots (cv. King Edward) were fed 200 µg D₅-cholesterol solubilised in Tween-80, after which leaves were analysed for endogenous and D₅-cholesterol by GC-MS. The chromatogram shows the molecular ions (M⁺) of D₅-cholesterol and endogenous cholesterol extracted from the total ion current during the same run. (PDF)

Figure S2. LC-MS/MS chromatogram from an analysis of potato leaves containing deuterium (D) -labelled SGA. Cut potato shoots (cv. King Edward) were fed 200 µg D₆-cholesterol solubilised in methyl-β-cyclodextrin after, which leaves were analysed for endogenous and D-SGA by LC-MS/MS. (A) D₆-α-solanine (left peak) and D₆-α-chaconine (right peak); and (B) endogenous α-solanine (left peak) and endogenous α-chaconine (right peak). (PDF)

Figure S3. GC-MS chromatogram from an analysis of hydroxysterols in potato leaves. Endogenous 26-hydroxycholesterol (retention time 43 min) in a potato shoot (cv. King Edward) analysed with GC-MS in single ion monitoring (SIM) mode. The peak matched an authentic 26-hydroxycholesterol standard both regarding retention time and characteristic ion fragments. (PDF)

Figure S4. Working model for the SGA biosynthesis from cholesterol. The reaction scheme is freely adapted from [16,20]. Occurrence of 26-hydroxycholesterol as an endogenous hydroxysterol in potato plants was demonstrated in the present study. (PDF)

Table S1. Levels of endogenous and deuterium (D) -labelled SGA. The SGA metabolites indicated in the table were analysed in upper and lower leaves of potato shoots (cv. King Edward) that had been fed 200 µg D-labelled sterols solubilised in Tween-80 or in methyl-β-cyclodextrin (MBD) for three or five weeks, and compared to water control samples (blanks). Each data point is based on one analysis of an extract made from two or three pooled shoots from independent plants. Levels of D-labelled SGA are corrected for the natural abundance of ¹³C where needed. ND, not detected. (PDF)

Table S2. Hydroxysterol levels in leaves of potato and Arabidopsis. Endogenous hydroxysterols were at two separate occasions measured in leaves of potato (cv. King Edward) and Arabidopsis (cv. Columbia), using 19-hydroxycholesterol as internal standard and quantification by GC-MS. Mean value ± range (n=2 separate analyses). ND, not detected. (PDF)

Author Contributions

Conceived and designed the experiments: EVP NN PD LJ FS. Performed the experiments: EVP NN PD RT. Analyzed the data: EVP NN PD. Contributed reagents/materials/analysis tools: RT PCD AB. Wrote the manuscript: EVP NN PD LJ FS.

References

- Benveniste P (2004) Biosynthesis and accumulation of sterols. *Annu Rev Plant Biol* 55: 429–457. doi:10.1146/annurev.arplant.55.031903.141616. PubMed: 15377227.
- Johnson DF, Bennett RD, Heftmann E (1963) Cholesterol in higher plants. *Science* 140: 198–199. doi:10.1126/science.140.3563.198. PubMed: 17819841.
- Itoh T, Tamura T, Mitsuhashi T, Matsumoto T (1977) Sterols of Liliaceae. *Phytochemistry* 16: 40–41.
- Itoh T, Tamura T, Matsumoto T (1977) 4-Desmethylsterols in the seeds of Solanaceae. *Steroids* 30: 425–433. doi:10.1016/0039-128X(77)90033-2. PubMed: 595039.
- Heftmann E (1983) Biogenesis of steroids in Solanaceae. *Phytochemistry* 22: 1843–1860. doi:10.1016/0031-9422(83)80001-6.
- Friedman M, McDonald GM (1997) Potato glycoalkaloids: Chemistry, analysis, safety, and plant physiology. *Crit Rev Plant Sci* 16: 55–132. doi:10.1080/713608144.
- Ginzberg I, Tokuhisa JG, Veilleux RE (2009) Potato steroidal glycoalkaloids: Biosynthesis and genetic manipulation. *Potato Res* 52: 1–15. doi:10.1007/s11540-008-9103-4.
- Sinden SL, Sanford LL, Webb RE (1984) Genetic and environmental control of potato glycoalkaloids. *Am Potato J* 61: 141–156. doi:10.1007/BF02854035.
- Valkonen JPT, Keskitalo M, Vasara T, Pietilä L (1996) Potato glycoalkaloids: A burden or a blessing? *Crit Rev Plant Sci* 15: 1–20. doi:10.1080/713608127.
- Korpan YI, Nazarenko EA, Skryshevskaya IV, Martelet C, Jaffrezic-Renault N et al. (2004) Potato glycoalkaloids: true safety or false sense of security. *Trends Biotechnol* 22: 147–151. doi:10.1016/j.tibtech.2004.01.009. PubMed: 15036866.
- Bömer A, Matti A (1924) Der Solaningehalt der Kartoffeln. *Z Unters Nahr Genussm* 47: 97–127.
- McCue KF, Shepherd LVT, Allen PV, Maccree MM, Rockhold DR et al. (2005) Metabolic compensation of steroidal glycoalkaloid biosynthesis in transgenic potato tubers: using reverse genetics to confirm the in vivo enzyme function of a steroidal alkaloid galactosyltransferase. *Plant Sci* 168: 267–273. doi:10.1016/j.plantsci.2004.08.006.
- McCue KF, Allen PV, Shepherd LVT, Blake A, Whitworth J et al. (2006) The primary in vivo steroidal alkaloid glucosyltransferase from potato. *Phytochemistry* 67: 1590–1597. doi:10.1016/j.phytochem.2005.09.037. PubMed: 16298403.
- McCue KF, Allen PV, Shepherd LVT, Blake A, Maccree MM et al. (2007) Potato glycoesterol rhamnosyltransferase, the terminal step in triose side-chain biosynthesis. *Phytochemistry* 68: 327–334. doi:10.1016/j.phytochem.2006.10.025. PubMed: 17157337.
- Itkin M, Rogachev I, Alkan N, Rosenberg T, Malitsky S et al. (2011) GLYCOALKALOID METABOLISM1 is required for steroidal alkaloid glycosylation and prevention of phytotoxicity in tomato. *Plant Cell* 23: 4507–4525. doi:10.1105/tpc.111.088732. PubMed: 22180624.
- Itkin M, Heinig U, Tzfadia O, Bhide AJ, Shinde B et al. (2013) Biosynthesis of antinutritional alkaloids in solanaceous crops is mediated by clustered genes. *Science* 341: 175–179. doi:10.1126/science.1240230. PubMed: 23788733.
- Tschesche R, Hulpke H (1967) Zur biosynthese vor steroid-derivaten im pflanzenreich.8. Biogenese von solanidin aus cholesterin. *Z Naturforsch B* 22: 791. PubMed: 4385314.
- Heftmann E, Weaver M (1974) 26-hydroxycholesterol and cholest-4-en-3-one, the first metabolites of cholesterol in potato plants. *Phytochemistry* 13: 1801–1803. doi:10.1016/0031-9422(74)85093-4.
- Tschesche R, Goossens B, Töpfer A (1976) Zur einfuehrung des stickstoffs und zum gemeinsamen vorkomen von 25(R) und 25(S)-steroidalkaloiden in Solanaceen. *Phytochemistry* 15: 1387–1389. doi:10.1016/S0031-9422(00)97122-X.
- Ohyama K, Okawa A, Moriuchi Y, Fujimoto Y (2013) Biosynthesis of steroidal alkaloids in Solanaceae plants: Involvement of an aldehyde intermediate during C-26 amination. *Phytochemistry* 89: 26–31. doi:10.1016/j.phytochem.2013.01.010. PubMed: 23473422.
- Bergensträhle A, Tillberg E, Jonsson L (1992) Regulation of glycoalkaloid accumulation in potato tuber discs. *J Plant Physiol* 140: 269–275.
- Schmitt P, Narula AS, Benveniste P, Rahier A (1981) Manipulation by 25-azacycloartenol of the relative percentage of C10, C9 and C8 side-chain sterols in suspension cultures of bramble cells. *Phytochemistry* 20: 197–201. doi:10.1016/0031-9422(81)85092-3.
- Bergensträhle A, Tillberg E, Jonsson L (1993) Effects of ethephon and norbornadiene on sterol and glycoalkaloid biosynthesis in potato tuber discs. *Physiol Plant* 89: 301–308. doi:10.1111/j.1399-3054.1993.tb00158.x.
- Bergensträhle A, Borgå P, Jonsson LMV (1996) Sterol composition and synthesis in potato tuber discs in relation to glycoalkaloid synthesis. *Phytochemistry* 41: 155–161. doi:10.1016/0031-9422(95)00554-4.
- Arqvist L, Dutta PC, Jonsson L, Sitbon F (2003) Reduction of cholesterol and glycoalkaloid levels in transgenic potato plants by overexpression of a type 1 sterol methyltransferase cDNA. *Plant Physiol* 131: 1792–1799. doi:10.1104/pp.102.018788. PubMed: 12692338.
- Gondet L, Bronner R, Benveniste P (1994) Regulation of sterol content in membranes by subcellular compartmentation of steryl-esters accumulating in a sterol-overproducing tobacco mutant. *Plant Physiol* 105: 509–518. PubMed: 12232218.
- Schaller H, Grausem B, Benveniste P, Chye M-L, Tan C-T, et al. (1995) Expression of the *Hevea brasiliensis* (H.B.K.) Müll. Arg. 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1 in tobacco results in sterol overproduction. *Plant Physiol* 109: 761–770.
- Behmer ST, Grebenok RJ, Douglas AE (2011) Plant sterols and host plant suitability for a phloem-feeding insect. *Funct Ecol* 25: 484–491. doi:10.1111/j.1365-2435.2010.01810.x.
- Silvestro D, Andersen TG, Schaller H, Jensen PE (2013) Plant Sterol Metabolism. Δ^7 -Sterol-C₅-Desaturase (STE1/DWARF7), $\Delta^{5,7}$ -Sterol- Δ^7 -Reductase (DWARF5) and Δ^{24} -Sterol- Δ^{24} -Reductase (DIMINUTO/DWARF1) Show Multiple Subcellular Localizations in Arabidopsis thaliana (Heynh) L. *PLOS ONE* 8(2): e56429.
- Beste L, Nahar N, Dalman K, Fujioka S, Jonsson L et al. (2011) Synthesis of hydroxylated sterols in transgenic Arabidopsis plants alters growth and steroid metabolism. *Plant Physiol* 157: 426–440. doi:10.1104/pp.110.171199. PubMed: 21746809.
- Chakraborty S, Shukla D, Jain A, Mishra B, Singh S (2009) Assessment of solubilization characteristics of different surfactants for carvedilol phosphate as a function of pH. *J Colloid Interface Sci* 335: 242–249.
- Stella VJ, He Q (2008) Cyclodextrins. *Toxicol Pathol* 36: 30–42.
- Klein U, Gimpl G, Fahrenholz F (1995) Alteration of the myometrial plasma membrane cholesterol content with β -cyclodextrin modulates the binding affinity of the oxytocin receptor. *Biochemistry* 34: 13784–13793. doi:10.1021/bi00042a009. PubMed: 7577971.
- Petersson EV, Arif U, Schulzova V, Krtková V, Hajšlová J et al. (2013) Glycoalkaloid and calystegine levels in table potato cultivars subjected to wounding, light, and heat treatments. *J Agric Food Chem* 61: 5893–5902. doi:10.1021/jf400318p. PubMed: 23692427.