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UPTAKE OF CARDENOLIDES FROM PHLOEM SAP INTO CROWN GALLS OF *DIGITALIS LANATA*

WOLFRAM PINKWART, BEATE DIETTRICH and MARTIN LUCKNER*

University Halle, Institute of Pharmaceutical Biology, Hoher Weg 8, 06120 Halle, Germany

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Key Word Index—*Digitalis lanata*; Scrophulariaceae; woolly foxglove; crown galls; transport; cardenolides.

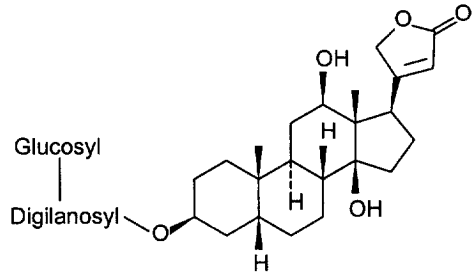
Abstract—Crown galls developing on leaves and stems of *Digitalis lanata* plants grown in the greenhouse contained digitalinum verum, glucodigifucoside, deacetyllanatoside C and verodoxin as the main cardenolides. Crown galls formed on leaves of axenic plants accumulated digitalinum verum, strospeside, lanatoside C and glucodigifucoside. These cardenolide profiles differed from those of the tumour-bearing leaves and stems, but resembled that of phloem sap. Crown gall tissue cultivated *in vitro* did not form cardenolides. However, crown galls *in situ* and crown gall tissue cultivated *in vitro* took up cardenolides. Digitalinum verum, a cardenolide characteristic for the phloem sap, was transported to, and accumulated in, the crown galls if administered to tumour-bearing leaves. It may therefore be speculated that the crown galls are sinks for cardenolides transported in phloem sap. The cardenolide-modifying enzymes acetyl-coenzyme A: digitoxin 15′-*O*-acetyltransferase, lanatoside 16′-*O*-acetylesterase, UDP-glucose: digitoxin 16′-*O*-glucosyltransferase and cardenolide 16′-*O*-glucohydrolase were present in crown gall tissue.

INTRODUCTION

Crown galls were induced on leaves and stems of *Digitalis lanata* by inoculation with different wild-type strains of *Agrobacterium tumefaciens*. The wide host range strain B6S3 pTi B6S3 [1] induced crown galls regularly with high rates [2]. With radioimmunoassays (RIAs) sensitive for digitoxin and digoxin derivatives it was shown that the crown galls contained cardenolides. The cardenolide content of the crown galls was smaller than that of the tumour-bearing leaves. In crown gall tissue cultivated *in vitro* rather small amounts of cardenolides were detected. It was therefore speculated that the cardenolides found in the crown galls were transported from the mother plant into the galls [2].

Christmann *et al.* [3] showed that cardenolides were transported in the phloem sap of *D. lanata* plants. They identified digitalinum verum, glucoverodoxin, glucodigifucoside, odorobioside G, deacetyllanatoside C and lanatoside C. This cardenolide spectrum differed markedly from that of *D. lanata* leaves [4].

It was the aim of the experiments described in this paper to examine whether crown galls are cardenolide sinks in *D. lanata* plants (i) by comparing the cardenolide spectrum of crown galls developing on leaves and stems with the cardenolides of these organs and the phloem sap, (ii) by examining the *de novo* synthesis of cardenolides in crown gall tissue *in vitro* and (iii) by investigating uptake of cardenolides into crown galls.



Digitalinum verum

RESULTS

Formation of crown galls

Crown galls were induced on leaves of juvenile *D. lanata* plants (rosettes) grown in the greenhouse during the first year of development and on stems of *D. lanata* greenhouse plants in the second year. Crown galls could be incited during the whole year. However,

* Author to whom correspondence should be addressed.

the rates of crown gall formation and the growth rate of the crown galls depended on the season. Development of crown galls was maximum in May/June and showed a second less pronounced peak in October/November on leaves of *D. lanata* greenhouse plants which were kept in the juvenile state by avoiding temperatures below 15° [5]. During the peak periods temperature in the greenhouse was abut 20–28°, i.e., optimum for crown gall formation [6].

The development of crown galls showed no seasonal variation on the leaves of axenic plants cultivated *in vitro* in climatized chambers due to constant growth conditions. However, the rates of tumour formation and growth were smaller if compared with greenhouse plants [7, 8]. For most experiments therefore crown galls developing on greenhouse plants were used.

Crown gall morphology

Microscopy showed that vascular tissue dominated at the site of connection between the tumour and the mother plant. Dense groups of xylem vessels directed to the outer layer filled the interior of the tumour. In the outer part of the crown galls randomly oriented individual xylem cells occurred in a matrix of partly lignified parenchyma-like cells. The tumour was covered by a layer of cork cells. Xylem (and probably phloem) formed a continuum between the mother plant and the tumour. This agrees with the results obtained with other plant species [9].

Cultivation of crown gall tissue in vitro

Tissue of crown galls formed on leaves of axenic plants was cultivated *in vitro*. Because the explants contained agrobacteria which were difficult to remove, they were cultivated in the presence of ampicillin. The transformed tissue showed good growth rates (doubling time about 10 days) in the absence of growth regulators in contrast to wound callus which died under these conditions. Initiation of growth of the crown gall tissue *in vitro* was facilitated by addition of growth regulators to the nutrient medium at the first passage *in vitro*. In the absence of growth regulators a process of self conditioning took place which lasted several weeks.

Octopine synthase activity in crown galls and regenerated plants

The enzyme octopine synthase was expressed in all transformed tissues examined (crown galls developing *in situ*, crown gall-derived tissue cultivated *in vitro* and plants regenerated from the crown gall tissue). The octopine synthase gene is part of the T-DNA of pTi B6S3. Expression of octopine synthase indicated that the T-DNA was integrated into the genome of *D. lanata*. During aging of regenerated plants the measurable enzyme activity became gradually smaller, probably due to inactivation of the appropriate gene.

Cardenolide profiles of crown galls, leaves and stems

HPLC analysis showed a cardenolide content of crown galls up to 3 μmol per g dry weight. The cardenolide content of the crown galls was lower than that of the tumour-bearing plant organs. This agrees with results obtained by Moldenhauer *et al.* [2]. The ratio of the cardenolide contents of the mother organ and the crown galls was highest for crown galls formed on leaves of greenhouse plants and lowest for crown galls developing on the leaves of axenic plants (Table 1).

The cardenolide spectra of crown galls did not reflect the different cardenolide profiles of the crown gall-bearing plant organs. Typical cardenolides of crown galls were diglycosides containing a digitalosyl residue. Digitoxosides and fucosides occurred in smaller quantities (Table 1). The main cardenolide was digitalinum verum (gitoxigenin glucosyldigitaloside). The concentration of this compound was higher in the crown galls than in the tumour-bearing organs (Table 1).

The highest cardenolide content was found in the leaves of *D. lanata* greenhouse plants (about 11 μmol per g dry weight). Lanatoside C was the main cardenolide in the leaves. This agrees with results of Wiegrebe and Wichtl [4]. Glucodigifucoside, deacetyllanatoside C, odorobioside G, lanatoside A and digitalinum verum also occurred in relatively high concentrations. Cardenolides of the C-series formed nearly 60% of the cardenolide amount. Dominating were digitoxose-containing tetraglycosides (Table 1).

Glucodigifucoside was the main cardenolide in stems of flowering greenhouse plants. More than 50% of the cardenolides belonged to the group of fucosides. Bulk compounds were diglycosides and cardenolides of the A-series (Table 1).

The cardenolide profile of leaves of axenic plants differed from that of greenhouse plants. Main cardenolide was glucodigifucoside. The leaves showed a high content of diglycosides, digitalosides and of compounds of the A- and B-series (Table 1).

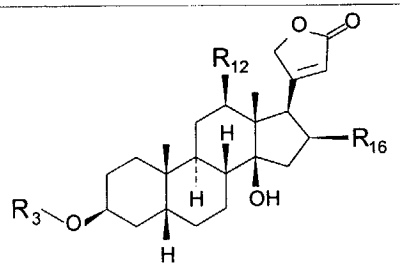
Absence of cardenolides in crown gall tissue grown in vitro

Crown gall tissue did not contain cardenolides in amounts measurable with HPLC after 1–2 weeks of cultivation *in vitro*. Cardenolides present in the crown gall tissue grown *in situ* used for inoculation were leaking out into the nutrient medium during the first days of growth. The absence of cardenolides in the transformed tissue cultivated *in vitro* disagrees with results obtained by Moldenhauer *et al.* [2] who, instead of HPLC, used more sensitive but less specific RIAs for cardenolide determination.

Uptake of cardenolides into crown gall tissue in vitro

Cardenolides were taken up from liquid medium by crown gall-derived tissue grown *in vitro*. However, the

Table 1. Cardenolide spectra of crown galls formed on leaves and stems of *D. lanata*



Cardenolides					Leaves of greenhouse plants[b]	Crown galls[b]	Stems of greenhouse plants[b]	Crown galls[b]	Leaves of axenic plants[b]	Crown galls[b]
Series	Compounds	R_3[a]	R_{12}	R_{16}						
A	Glucodigifucoside	Glc-Fuc-	-H	-H	1.71	0.40	2.04	0.44	0.39	0.10
	Odorobioside G	Glc-Dtl-	-H	-H	0.62	0.17	0.42	0.08	0.05	
	Purpureaglycoside A	Glc-Dx-Dx-Dx-	-H	-H	0.26	0.18			0.07	0.05
	Lanatoside A	Glc-AcDx-Dx-Dx-	-H	-H	0.43	0.03	0.18	0.06	0.14	
B	Digitalinum verum	Glc-Dtl-	-H	-OH	0.40	0.77	0.40	0.51	0.36	0.67
	Strospeside	Dtl-	-H	-OH	0.15	0.27	0.04	0.09	0.29	0.29
	Lanatoside B	Glc-AcDx-Dx-Dx-	-H	-OH	0.31					
C	Deacetyllanatoside C	Glc-Dx-Dx-Dx	-OH	-H	0.81	0.38	0.19	0.25	0.05	
	Lanatoside C	Glc-AcDx-Dx-Dx-	-OH	-H	5.38	0.26	0.50	0.12	0.31	0.29
	α-Acetyldigoxin	αAcDx-Dx-Dx-	-OH	-H	0.24	0.01			0.05	0.03
	β-Acetyldigoxin	βAcDx-Dx-Dx-	-OH	-H	0.02	0.05				
E	Glucoverodoxin	Glc-Dtl-	-H	-OCHO	0.31	0.11				
	Verodoxin	Dtl-	-H	-OCHO	0.29	0.35	0.24	0.17		
	Lanadoxin	Dx-	-H	-OCHO	0.04	0.02				
Sum					10.97[c]	3.00[c]	4.00[d]	1.72[d]	1.71[e]	1.43[e]
Monoglycosides	(%)				4.4	21.3	6.8	15.2	17.0	20.3
Diglycosides	(%)				27.7	48.3	71.4	59.8	46.8	53.9
Triglycosides	(%)				2.4	2.0	0	0	2.9	2.1
Tetraglycosides	(%)				65.5	28.3	21.8	25.0	33.3	23.8
Fucosides	(%)				15.6	13.3	51.0	25.6	22.8	7.0
Digitalosides	(%)				16.1	55.7	27.4	49.4	40.9	67.1
Digitoxosides	(%)				68.3	31.0	21.7	25.0	36.3	25.9
A series	(%)		-H	-H	27.5	26.0	66.0	33.7	38.0	10.5
B series	(%)		-H	-OH	7.8	34.7	11.0	34.9	38.0	67.1
C series	(%)		-OH	-H	58.8	23.3	17.3	21.5	24.0	22.4
E series	(%)		-OH	-HCHO	5.8	16.0	6.0	9.9	0	0

[a] Glc = glucosyl residue; Dx = digitoxosyl residue; AcDx = acetyldigitoxosyl residue; Dtl = digitalosyl residue; Fuc = fucosyl residue.
[b] μmol g^{-1} dry weight, analysis of 12 parallel samples (standard deviation ±39.3%).
[c] Ratio of cardenolide content: 3.7:1.
[d] Ratio of cardenolide content: 2.3:1.
[e] Ratio of cardenolide content: 1.2:1.

Table 2. Accumulation of digitalinum verum and β-methyldigoxin in crown galls after injection into veins of tumour-bearing leaves

Time after injection [h]	Digitalinum verum [μmol g^{-1} dry weight]		β-Methyldigoxin [μmol g^{-1} dry weight]	
	Leaf	Crown gall	Leaf	Crown gall
0	0.52	0.90	0	0
2.5	1.50	1.78	9.32	0.03
24.0	1.01	3.17	4.57	0.68

rates were slower than those obtained with untransformed *D. lanata* cells (strain W 1.4). This was caused probably by the more compact growth of the cell colonies in the crown gall tissue cultures. Both primary cardenolides (lanatoside C, deacetyllanatoside C, lanatoside A) and secondary cardenolides (digitoxin, digoxin) added to the culture medium were accumulated in crown gall cells. Lanatoside C and digoxin were transformed to deaceytllanatoside C. The glucosylation of digoxin is a widespread reaction in *D. lanata* cell cultures [10, 11]. Also deacetylation of lanatoside C has been found frequently [12, 13]. In contrast to cells of strain W 1.4, the crown gall cells did not form lanatoside C from digoxin. This agrees with the lack of acetyl-coenzyme A: digitoxin 15′-*O*-acetyltransferase activity in the crown gall-derived cell lines.

Transport of administered cardenolides to crown galls in situ

The uptake of cardenolides into crown galls *in situ* was examined with isotope-labeled cardenolides. The compounds were applied (i) to the surface of leaves of greenhouse plants in the neighbourhood of developing crown galls, (ii) through the stalk of excised leaves carrying crown galls, or (iii) by microinjection into the veins of crown gall-bearing leaves. Highest accumulation of fed cardenolides within short time periods was found after microinjection. Digitalinum verum was the cardenolide accumulated best. At 24 h after microinjection, the concentration of digitalinum verum in the crown galls was about three times higher than in the leaves, in contrast to β-methyldigoxin, a semisynthetic cardiac glycoside used as control, the amount of which was still 6–7 times higher in the leaves than in the crown galls (Table 2). Cardenolides occurring in the crown galls *in situ* in low concentration were accumulated to a smaller extent than digitalinum verum.

Enzymes transforming cardenolides in crown gall tissue

The activities of acetyl-coenzyme A: digitoxin 15′-*O*-acetyltransferase (DAT), lanatoside 16′-*O*-acetylesterase (LAE), UDP-glucose: digitoxin 16′-*O*-glucosyltransferase (DGT) and cardenolide 16′-*O*-glucohydrolase (CGH) were examined in enzyme preparations obtained from crown galls and leaves of *D. lanata* greenhouse plants. The activities of LAE and CGH in the enzyme preparations from crown galls were similar or even higher than in enzyme preparations from leaves, whereas the activity of DAT was rather small. DGT activity was not measurable in enzyme preparations from crown galls (Table 3).

Crown gall cells cultivated *in vitro* for several

Table 3. Activities of cardenolide-modifying enzymes in crown galls, crown gall-derived cell lines and leaves of *D. lanata*

Tissue/Organ	DAT[1]	LAE[2]	DGT[3]	CGH[4]
Crown galls *in situ*	3.7±0.3	32.8±3.15	0	1500±101.5
Crown gall tissue cultivated *in vitro*	0	20.9±1.80[5]	8.0±3.50	0
Young leaves	37.3±12.1	15.0±1.88	7.3±0.91	1128±116.4
Middle leaves	9.0±1.1	14.1±4.36	0.9±0.12	1578±282.3
Old leaves	0	0	0.4±0.07	1505±141.3

Enzyme activity: μkat kg^{-1} protein, analysis of 6 parallel samples (standard deviation ±15%).
[1] DAT = acetyl-coenzyme A: digitoxin 15′-*O*-acetyltransferase.
[2] LAE = lanatoside 16′-*O*-acetylesterase.
[3] DGT = UDP-glucose: digitoxin 16′-*O*-glucosyltransferase.
[4] CGH = cardenolide 16′-*O*-glucohydrolase.
[5] Enzyme activity found only in 2 of 8 cell lines investigated.

months showed DGT activity in all cell lines investigated. LAE activity was measurable in a few cell lines only. DAT and CGH activities were absent. High CGH activities were determined in leaves of *D. lanata* greenhouse plants. DAT, LAE and DGT activities were highest in young leaves and decreased during aging of the leaves. DAT and LAE activities were not measurable in old leaves (Table 3).

DISCUSSION

Crown galls as cardenolide sink

There were two results indicating that the cardenolides found in crown galls developing *in situ* originate from the mother plant: (i) crown gall tissue grown *in vitro* was free of cardenolides and (ii) crown galls *in situ* and crown gall tissue cultivated *in vitro* were able to take up cardenolides. The cardenolide spectrum of the crown galls differed from that of the tumour-bearing leaves or stems. Digitalinum verum was the main cardenolide in the crown galls irrespective of the cardenolide profile of the crown gall-bearing organs (Table 1). Transport of the bulk cardenolides of leaf or stem tissue into crown galls was therefore unlikely to occur.

Crown galls are sinks of nutrients and water [14]. The xylem vessels present in the crown galls are directly connected to those of the mother plant [9, 15] and a similar continuum is expected relative to the phloem cells. The cardenolide spectrum of the crown galls resembled that of the phloem sap of *D. lanata* [3]. This indicated a specific transport of cardenolides into the crown galls. Digitalinum verum, a typical phloem sap cardenolide [3] accumulated in crown galls if administered into the vein of leaves on which tumours were developing. After a few hours its concentration exceeded that of the leaf tissue in contrast to β-methyldigoxin used as control which was not transported in the phloem (Table 2). There is evidence for symplastic phloem unloading of nutrients in the crown galls [16]. Probably also the cardenolides present in the phloem sap of *D. lanata* were released in this way causing the accumulation of phloem-specific cardenolides in the tumours.

Crown galls contained high activities of the enzyme CGH (Table 3). This might be the reason for the occurrence of the deglucosylated cardenolides strospesid and verodoxin in crown galls which are present in their glucosylated form in the phloem sap [3]. Boettingheimer and Kreis [17] have shown that in addition to CGH, which deglucosylates preferentially tetrasaccharides of the lanatoside type, a second glucohydrolase exists in *D. lanata* leaves which predominantly hydrolyzes cardenolide disaccharides (CGH II). It will be tested in further experiments whether CGH II is present in crown gall tissue. Expression of this enzyme may explain the occurrence of deglucosylated disaccharides but the absence of deglucosylated tetrasaccharides in the crown galls.

EXPERIMENTAL

General

Agrobacteria: *A. tumefaciens* strain B6S3 pTi B6S3 [18] was used for infection of *D. lanata*. The agrobacteria were cultivated on a solidified YEB medium as described by Van Larebeke *et al.* [19].

Digitalis lanata

(a) Greenhouse plants: Plants of *D. lanata* cv. *Dresdener* were grown in the juvenile state as rosettes in the greenhouse at temperatures not lower than 15°. They formed elongated stems after vernalization in the second vegetation period. (b) Axenic plants: Seeds were treated with EtOH (3 min), rinsed with H_2O and sterilized with a soln of 5 mmol tosylchloramide sodium, 90 ml H_2O, 10 ml 1 M HCl and 1 drop of Tween 80. After 30 min they were again rinsed with H_2O. Axenic seedlings were grown on Nml without hormones and solidified with agar. After 2 weeks the plantlets were cultivated in Nml without hormones and solidified with perlite®. Cultivation was carried out at 25° and illumination for 16 h with fluorescent tubes (2 W m^{-2}). (c) Axenic shoots: Shoots of clone 15 were grown as described by Mertinat *et al.* [5].

Infection of D. lanata *plants*

Juvenile plants were inoculated with agrobacteria 5–6 months after sowing. Stems of flowering plants were infected at the beginning of development. Leaves and stems were wounded with a lancet (greenhouse plants: maximum 10 incisions per leaf and 5 incisions per stem; axenic plants: 3 incisions per plant) and inoculated with 10–20 μl *Agrobacterium* suspension. Axenic plants were cultivated on Nml solidified with agar. The nutrient agar was covered with ampicillin solution (250 μM).

Nutrient media

Nutrient medium 1 (Nml) was prepared according to Moldenhauer *et al.* [2].

Cultivation of transformed and untransformed cell lines of D. lanata

(i) Transformed cell lines: Crown gall tissue developing on axenic plants was removed 2–3 months after infection and cultivated in Nml containing 5 μM 2,4-D and 0.1 μM kinetin in the presence of 250 μM ampicillin at 23° and agitation (60 rpm). After 1 week the tissue was transferred to phytohormone-free Nml containing 250 μM ampicillin. The cells were cultivated in this medium for several years. (ii) Wound callus: Excised disks of leaves were cultivated for two months on Nml containing 5 μM 2,4-D and 0.1 μM kinetin which was solidified with agar. The developing

wound callus was removed and cultivated as described for the crown gall tissue, but without ampicillin. The tissue died after 1–2 weeks of cultivation in the hormone-free medium. (iii) Cells of the untransformed strain W 1.4 were cultivated as described by Kreis *et al.* [20].

Opine synthase

Activity of this enzyme was measured in crown galls according to Otten and Schilperoot [21] and in transformed cell lines and plants according to Aerts *et al.* [22].

Uptake of cardenolides into crown gall tissue cultivated in vitro

The experiments were performed as described in Refs [10] and [11].

Uptake of cardenolides into crown galls in situ

(i) Leaves were rinsed with an aq. soln of Tween 80 (3 drops per 100 ml). Generally tritiated cardenolides (lanatoside A, digoxin and digitoxigenin) were dissolved in MeOH. A few drops of the methanolic soln were spread repeatedly with a brush at the leaf surface near developing crown galls which were surrounded by a ring of silicon grease to prevent direct contact with the cardenolide solution. After 48 h the leaves were harvested, briefly rinsed with MeOH to remove unabsorbed cardenolides and dried. (ii) Leaves bearing crown galls were dipped into 0.5 ml of an aq. soln containing 100 μg of cardenolides. After absorption of the cardenolide soln, 0.5 ml of H_2O was added. (iii) The cardenolides (3 mg) were dissolved in 125 μl MeOH. The soln was diluted with H_2O to 400 μl, thoroughly mixed and then 5–10 μl per leaf were administered using a syringe with a fine needle into the main veins 1–2 cm apart from developing crown galls. The leaf lamina turned dark green near the site of injection due to invasion of the soln into the intercellular cavities.

Enzymes of cardenolide transformation

Activities of the enzymes acetyl-coenzyme A: digitoxin 15′-*O*-acetyltransferase, lanatoside 16′-*O*-acetylesterase, UDP-glucose: digitoxin 16′-*O*-glucosyltransferase and cardenolide 16′-*O*-glucohydrolase were determined as described by Refs [23], [24] and [25], respectively.

Analysis of cardenolides

Dried plant material (150 mg) containing 600 μg β-methyldigoxin as internal standard were extracted with 15 ml MeOH (70%) under reflux on a water bath for 10 min. The extract was centrifuged for 5 min at 5000*g* and then 5 ml lead acetate soln (15%) were added followed after 1 min by 5 ml monosodiumphosphate soln (4%). The mixt was then centrifuged again. An aliquot of the resulting soln was extracted by solid-phase as described by Ref. [4]. HPLC analysis was carried out with MeCN (84%) and H_2O as liquid phases with a flow rate of 1.0 ml min^{-1}. Gradient: MeCN: start = 20%, 32 min = 32%, 45 min = 40%, 55 min = 50%, 57 min = 52%, 59 min = 55%, 63 min = 92%, 65 min = 92%, 70 min = 20%, 72 min = stop. A Hypersil ODS RP-18 (5 μm) column (250.4 mm) was used as solid phase in combination with a precolumn (8.4 mm) filled with the same material. The columns were equilibrated at 40°. Spectra were measured with a variable wavelength detector. Radioactivity was determined with the monitor LB 506 C-1 containing the solid scintillation measuring cell YG400U4D (Berthold).

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