[Frontiers in Bioscience E2, 1196-1210, June 1, 2010]

Induced endogenous autotoxicity in *Camptotheca*

Shiyou Li, Ping Wang, and Wei Yuan

National Center for Pharmaceutical Crops, Arthur Temple College of Forestry and Agriculture, Stephen F. Austin State University, Nacogdoches, TX 75962, USA

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1. ABSTRACT

Are plants always immune to their endogenous toxic secondary metabolites? Without disturbance, fast-growing *Camptotheca* plants can avoid poison by its endogenous camptothecin (DNA topoisomerase I inhibitor) at more than 10 times higher than the fatal concentration of exogenous application to the plant. Pruning has been long known to promote lateral growth; however, here we report that auxin-reducing pruning can induce endogenous autotoxicity in *Camptotheca*: dramatic deviations from normal morphogenesis, including serrated or lobed leaves, disturbed phyllotaxis, and fasciated stems. The abnormal morphogenesis appears correlatively with the elevated camptothecin contents following decapitation pruning. Plants resume their normal morphogenesis when camptothecin is reduced to natural levels after stress discontinuation. Exogenously applied auxin restores a dwarf mutant of high camptothecin yield to its parent type of lower yield, suggesting that auxin may be a triggering factor for the observed autotoxicity. Autotoxicity induced by endogenous compounds has not been reported before.

2. INTRODUCTION

The potential for autotoxicity by secondary metabolites in living plants has been hypothesized to exist for at least three decades (1, 2). Reports on exogenous autotoxicity (allelopathic effects of secondary metabolites to the parent plants by exogenously-applied metabolites) are numerous, and the terms "autointoxication", "autotoxicity", and "intraspecific allelopathy" have been used in many weed and crop plants for the inhibition of the growth of other individuals of its own kind through the release of toxic chemicals into the environment (3, 4). In contrast, endogenous autotoxicity (toxicity *in planta* by endogenous secondary compounds) has been poorly understood because evidence is scarce due to experimental difficulties (5, 6).

Is a plant ever self-poisoned by its endogenous toxic secondary metabolites? It is reported that damage-induced and elevated essential oils caused reductions in photosynthetic capacity in parsley, parsnip, and lemon (6). But Baldwin and Callahan (1993) found that exogenously-

fed nicotine did not poison photosynthetic capacity or cause leaf damage to nicotine-producing tobacco (*Nicotiana sylvestris* Speg. & Comes) because the damage-induced nicotine synthesis increased the tolerance of tobacco (5). In fact, it has been widely reported or believed that plants can avoid self-toxicity by their endogenous cytotoxic metabolites (5-8). Therefore, studies in endogenous autotoxicity have been primarily focused on plant avoidance of autotoxicity and detoxification mechanisms. For example, extracellular excretion, vacuolar sequestration, vesicle transport, extracellular biosynthesis, target mutations, and accumulation of metabolite in a non-toxic form, have been proposed (7, 8).

Alkaloid camptothecin (CPT) is an uncompetitive inhibitor of topoisomerase I (TOP1), a DNA replication enzyme, and stops cell division in humans, animals, insects, and plants (9-11). To date, two CPT drugs have been approved by FDA for cancer treatment with 14 CPT derivatives in clinical trials. CPT also shows antiviral and antifungal activities (11). *Camptotheca* Decaisne (Nyssaceae), known as the happytree, with three relic species restricted to southern China, is the major natural source of CPT (12). Since its antitumor activity was discovered in 1958 (13), there have been extensive studies in chemical and medical aspects of the tree and CPT. However, the *in vivo* role of CPT in *Camptotheca* plants remains elusive. With high CPT content in young leaves of *Camptotheca* (up to 0.12% on the basis of fresh weight, about tens of times higher than the effective cytotoxic concentration for external application), trees can still grow to 3 m in height annually with normal morphogenesis under natural conditions (14). It was thus believed that *Camptotheca* and other CPT-producing plants can avoid self-toxicity by its biosynthetic CPT (7, 8). Sirikantaramas *et al.* have recently found that the TOP1 mutations in *Camptotheca acuminata* Decaisne and other CPT-producing plants rendered the plants completely resistant to CPT (7, 8). Considering the CPT-insensitivity of CPT-producing plants in cell culture and the resistance of the yeast cells expressing TOP1 from CPT-producing plants to CPT, these authors concluded the TOP1 mutations are a self-resistance mechanism of CPT-producing plants (8).

Our *in planta* investigations revealed that *Camptotheca* plants cannot avoid toxicity of CPT when it becomes available. Like CPT-nonproducing red beans, *C. acuminata* seedlings were poisoned by externally-applied CPT to apical buds. Some treated plants in both red bean (*Phaseolus vulgaris* L.) and *C. acuminata* demonstrated dramatic morphological deviations from normal development. More interestingly, a similar abnormal morphogenesis displayed in *C. acuminata* plants due to the increased level of the endogenous CPT following decapitation (pruning of apical buds of the central stems or the stems). The mechanism of this induced endogenous autotoxicity is not elucidated, but it could involve TOP1s, the only known target of CPT. Exogenously applied auxin restoration of an auxin deficient mutant also suggests that auxin deficiency as a possible triggering factor for endogenous autotoxicity.

Decapitation pruning has been a common practice used to improve plant growth, form, or productivity. However, the abnormal morphogenesis observed in *Camptotheca* has not been reported in any plant species before. It is probably because such an induced autotoxicity may occur only in some specific group of plants (e.g., plants that produce secondary metabolites toxic to DNA TOP1). Another reason is that existing autotoxicity studies have been primarily measured by either plant photosynthesis or *in vitro* cell viability (5, 6). Measurement of photosynthetic capacity does not involve morphological analysis, whereas any *in vitro* assays using cell culture do not demonstrate any abnormal morphogenesis as observed in *in planta* experiments. Based on observations in *Camptotheca*, we speculate that plants may be able to balance their metabolites to avoid self-toxicity during normal development. However, plants could be aberrant from the balance due to severe environmental stresses and thus have endogenous autotoxicity which includes phenotypic abnormalities. The observed induced autotoxicity in *Camptotheca* not only presents new direction for *in vivo* biosynthesis of toxic secondary metabolites in plants but also provides a basis to develop strategies to enhance biosynthesis and derivatization of target compounds. The discovery of CPT-induced abnormal morphogenesis in *Camptotheca* plants may provide useful information for better understanding possible mutagenesis and mechanisms of resistance to CPT drugs in human cancer therapy.

3. MATERIALS AND METHODS

3.1. Decapitation pruning

Experimental plants of *C. acuminata* for pruning experiment are 6-year-old trees grown in the field in Nacogdoches, Texas, USA. The seeds of these plants were collected from a single parent tree in China. Five plants were used as control and three intact clippings of young tissues (stems with leaves; ≤ 2 weeks) were collected monthly for each plant from March to October. The other five plants were treated with decapitation pruning (trunk removal at about 40 cm above ground) in late March. For each treated plant, three intact young clippings were collected monthly in late March, and from May to October (no significant growth in April). For all new growth observed during every month (from March to May of the following year), leaf margin (entire, serrate, or lobe), leaf shape, phyllotaxis, and stem status were recorded for each of the 10 plants, separately. The harvested clippings in both pruning and control experiments were analyzed for CPT content. CPT analysis was used as the established method at the National Center for Pharmaceutical Crops of Stephen F. Austin State University (15). Only morphological traits were analyzed in *C. yunnanensis* Dode following the same treatments and under the same growing conditions.

3.2. Exogenous application of CPT

Pure CPT (>95% in purity), previously isolated from *C. acuminata* leaves was dissolved in DMSO and water (1:9). 180 3-month-old *C. acuminata* seedlings from the same seed source were divided into six groups with 30 plants each group. The treatments were conduced on April

04, 2006. The first group had no treatment, the apical bud of each plants in the second was treated with DMSO (10%) only, the apical bud of each plant in the other four groups were treated with 0.001, 0.005, 0.01, and 0.02%, respectively. To evaluate the toxicity of CPT to CPT-nonproducing plants, red bean was investigated. Seeds of red bean were soaked in CPT at the concentrations of 0 (10% DMSO), 0.001, 0.005, 0.01, and 0.02% with 10 seeds in each petri dish and three replicates for each treatment. At 48 h, radicle length of each seed was measured and then the seeds were sown in pots separately. Morphological analysis of leaves and stems was conduced one month after the treatment.

3.3. External application of auxin

'CT168' is a dwarf mutant of the semi-dwarf cultivar of *C. lowreyana* Li 'Katie' (12). The mutant is characterized by small heterogeneous leaves, reduced internodes, and profuse branching. Mature 'CT168' grows to 1 m in height while mature 'Katie' reaches 3 m. Indole-3-butyric acid (IBA) (0.1%) was applied basally to 120 cuttings to induce roots under a mist system in the greenhouse. Plant development, including the status of stems, leaves, and roots, was examined every two weeks following treatment.

3.4. Isolation of compounds

Leaves, bark, fruits, and roots of *C. acuminata* were collected from untreated mature trees grown in Nacogdoches, Texas. Air-dried plant material of each part was ground to a coarse powder and percolated with 95% ethanol five times. Removal of the solvent under a vacuum at 80ºC yielded an EtOH extract for isolation. (1) For leaves: the leaf ethanol extract was dissolved in MeOH/H_2O (9:1) and then partitioned successively with hexane and $CHCl_3$ to yield hexane extract, $CHCl_3$ extract, and MeOH/H_2O extract. The extract was then fractionated on a silica gel column using $CHCl_3$/EtOAc (9:1 and 4:1) and then $CHCl_3$/MeOH (9:1, 4:1, and 2:1). The fractions were further separated by preparative TLC (silica gel) or chromatographed on a silica gel column. (2) For bark: the bark extract was dissolved in MeOH/H_2O (9:1) and then partitioned successively with hexane and $CHCl_3$ to yield hexane extract, $CHCl_3$ extract, and MeOH/H_2O extract. Then the extract was chromatographed on a silica gel column. (3) For fruits: the fruit extract was dissolved in MeOH/H_2O (9:1) and defatted with hexane to afford hexane extract and MeOH/H_2O extract. Each extract was subjected to column chromatography on silica gel to yield pure compounds. (4) For roots: the extract was chromatographed over an ODS column or a silica gel column or purified by preparative TLC (silica gel).

3.5. Cytotoxicity assays

Human prostate cancer (PC-3) (obtained from The University of Texas M. D. Anderson Cancer Center, Houston was maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum and antibiotics (1,000 units/mL penicillin-streptomycin solution, Hyclone). Cells were grown at 37°C and 5% CO_2 in humidified air. The isolated compounds from *C. acuminata* were tested at concentrations of 25 μM with three replications. Cells were inoculated into 96 well microtiter plates in 100 μL at plating densities of 6,000 cells/well (measured by Z2 Coulter Counter of Beckman). Cell growth and viability was measured by WST-8 (water soluble tetrazolium) assays following a standard protocol (Cell Counting Kit-8, CCK-8, Dojindo Molecular, Maryland). Cancer drug Doxorubicin was used as the positive control in the cell-based assays.

3.6. DNA TOP1 assays

Pure compounds isolated from *C. acuminata* leaves were evaluated for their ability to inhibit DNA TOP1 activity following the protocol described by Webb and Ebeler (16). This protocol allows for the detection of compounds with the ability to inhibit TOP1 through intercalation, inhibition of relaxation activity, or by poisoning. For the poisoning section of the assay, the known TOP1 poison CPT was used as a control and ethidium bromide was used as a DNA intercalation control. Luteolin was also used as an enzyme inhibition control. Each compound was tested at the concentration of 312 μM. For the quantitative determination of TOP1 inhibitory activity, photographic negatives of gel were densitometrically scanned by Molecular Imager® Gel Doc™ XR System (Bio-Rad). The area representing supercoiled DNA, migrating as a single band at the bottom of the gel, was determined.

3.7. Statistical analysis

Results are presented as mn ± s.d. Statistical analyses were performed using one-way ANOVA and Student's *t*-test. A value of $P \leq 0.05$ was considered to be statistically significant.

4. RESULTS

4.1. Abnormal morphogenesis and elevated CPT levels following decapitation

Without disturbance, *C. acuminata* has exclusively serrate leaves during its juvenile stage (e.g., one-year-old) and completely entire leaves when the tree becomes mature (e.g., 6-year-old) (Figure 1*A*, *B*; Figure 2*A*). Decapitation treatments in March alternated almost 100% newly emerged leaves in mature trees of *C. acuminata* to non-entire leaf margins in May: 50-76.7% serrate and 23.3-50% in other abnormal forms (e.g. bidentate, bifid, tridentate, and trifid) (Figure 1*C-I*; Figure 2*A*). Non-serrate abnormal leaves during new growth gradually disappeared until August while the serrate form among new leaves gradually decreased to 41.5% in October. Decapitation also caused disturbed phyllotaxis and reduced stems (Figure 1*H-J*). The treated trees restored their normal entire leaves and phyllotaxis in the latter part of the growing season and thereafter. Following decapitation, CPT contents in plants increased by 337%, 122%, and 229% in May, June, and August, respectively, and fell back to control levels in October, a trend that is in contrast to the plants under control conditions during the same months (Figure 2*B*). Similar patterns were also observed in decapitated *C. yunnanensis* and *C. lowreyana* trees. The abnormal leaf forms and phyllotaxis have not been observed in any wild type *Camptotheca* plants without

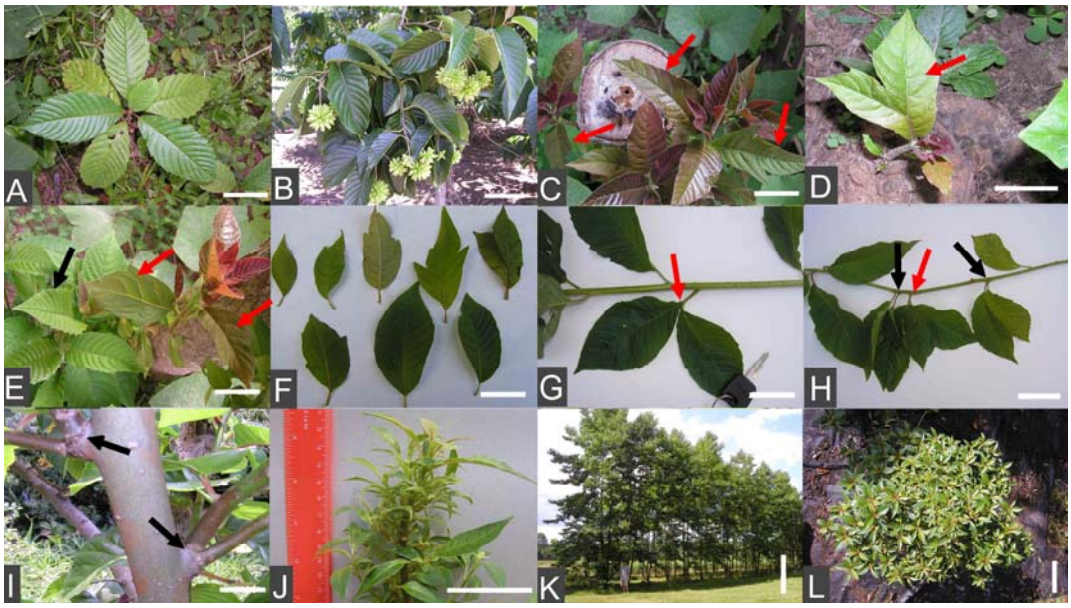

Figure 1. Normal phenotype of wild-type *C. acuminata* (A, B, and K), abnormal morphogenesis of *C. acuminata* induced by auxin-deficiency following decapitation pruning (C to I), and genetic mutants of *C. lowreyana* (J and L). (A) Serrated leaves of young seedlings of wild-type *C. acuminata*. (B) Entire leaves of mature trees of wild-type *C. acuminata*. (C-E) New lobed or serrated leaves of *C. acuminata* induced by decapitation pruning 5 weeks after treatment (red arrows for lobed forms and black arrow for serrated ones). (F) Comparison of abnormal leaves induced by decapitation pruning with leaves of wild-type *C. acuminata* (black arrows). (G) Bifid leaf of *C. acuminata* induced by decapitation pruning (red arrow). (H) Disturbed phyllotaxis (black arrow indicates two leaves growing together) and trifid leaf (red arrow) of *C. acuminata* induced by decapitation pruning. (I) Fasciated stems of *C. acuminata* induced by decapitation pruning (black arrow indicates the development of two lateral stems from a node). (J) Reduced internodes of dwarf *C. lowyerana* cultivar 'CT168'. (K) Wild-type *C. acuminata* trees can grow up to 45 meters in height. (L) Fasciated stems, reduced internodes, small and serrated/lobed leaves, and disturbed phyllotaxis in the cultivar 'CT168' of auxin deficiency. (a to j: bar=5 cm; k: bar=10 cm; l: bar=2 m).

disturbance, based on observation of more than 60,000 seedlings and over 1,000 mature trees from 1993 to 2005.

4.2. Abnormal morphogenesis following exogenous application of CPT

All apical buds of the three-month old seedlings of *C. acuminata* were poisoned by direct exogenous application of CPT at concentrations of 0.01% and 0.02%. After four weeks, abnormal morphogenesis of new leaves and stems was observed in each of the *Camptotheca* plants in which apical buds were killed by the poison (Figure 3). After 48 h of soaking treatment, red beans treated with CPT treatments displayed significant slow growth. For example, the average radicle length of the beans treated with 0.02% CPT was only 37.6% of the untreated seeds. Similar to *C. acuminata*, six plants of the total 36 red bean seedlings germinated from the seeds soaked in 0.02% CPT displayed significant abnormal morphogenesis (with one to four abnormal leaves per plant). The poisoned red bean plants had bifid or lobed leaves as those observed in the poisoned or decapitated *Camptotheca* plants (vs. normal trifid and entire leaves in untreated bean plants).

4.3. Cytotoxicity and DNA TOP1 activity of isolated compounds

Totally, 25 compounds were isolated from *C. acuminata*, including 17 compounds isolated from leaves, 8 from bark, 4 from fruits, and 16 from roots (Table 1) (17-35). Of these compounds, CPT and its five minor derivatives poisoned TOP1 and are cytotoxic. Five flavonoids, namely quercetin, hyperoside, isoquercitrin, trifolin, and astragalin inhibited the free TOP1 enzyme but they are not cytotoxic. The other test compounds are inactive in both TOP1 and cytotoxicity assays (Table 1).

5. DISCUSSION

5.1. Endogenous autotoxicity induced by elevated CPT levels

TOP1 is an enzyme involved in DNA replication, transcription, and recombination and thus, plays a critical role in cell proliferation (36). The TOP1 function has been shown to be essential to the growth and development of multicellular organisms (36). The enzyme is known to be the only known target of CPTs, which transform TOP1 into a DNA-damaging agent (9). During the last two decades, there have been extensive studies on the inhibitory effect of CPT on TOP1 in cancer biology and many eukaryotes, but little research has been done on plants. Since the first identification of plant TOP1 enzyme activity in wheat germ in 1981 (37), studies have been primarily focused on biochemical characterization and gene cloning (10). The toxicity of endogenous CPTs in living plants has been poorly understood (8).

Table 1. Compounds isolated from *Camptotheca acuminata*

No.	Compound Name	Concentrations (w/w, dry wt)						Bioactivity	
		Leaves	Stem Bark	Stem Wood	Fruit	Roots	Roots*	DNA TOP1 IC50 (μM)	Inhibition% of PC-3 at 25 μM
1	camptothecin	0.054-0.231%	0.032-0.067%	0.008-0.014%	0.084-0.231%	0.009-0.034%	0.03-0.07%	P: 0.52 (Ref, 17)	100
2	10-hydroxycamptothecin	<0.005%	<0.005%	Ref, 13	<0.005%	<0.005%	0.01%	P: 0.11 (Ref, 17)	100
3	10-hydroxy-20-deoxycamptothecin				Ref, 18				
4	10-methoxycamptothecin		Ref, 19	Ref, 19	Ref, 20		<0.001%	P: 0.30 (Ref, 17)	100
5	10-methoxy-20-*O*-acetylcamptothecin						<0.001%		70.4±9.9
6	11-hydroxycamptothecin				Ref, 21, 22				
7	11-methoxycamptothecin				Ref, 20				
8	18-hydroxycamptothecin		Ref, 23		Ref, 24			P: 9.6 (Ref, 17)	
9	20-hexanoylcamptothecin		Ref, 25						
10	20-*O*-acetylcamptothecin	<0.001%					<0.001%		100
11	20-hexanoyl-10-methoxycamptothecin		Ref, 25						
12	20-deoxycamptothecin		Ref, 25		Ref, 20, 26		<0.001%		71.2±9.0
13	20-*β*-D-glucopyranosyl 18-hydroxycamptothecin						<0.001%	N	N
14	benz [6, 7] indolizino [1, 2-b] quinoline-11 (13H)-one, 7-aldehyde-						<0.001%	N	N
15	[3S-(3α,4β,4aα,5aβ)]-4-ethenyl-3-(β-D-glucopyanosyyloxy)-4,4a,5,5a,6,12-hexahydro-3H-pyranol[3',4':6,7] indolizino[1,2-b]quinoline-11,14-dione		Ref, 23						
16	19-hydroxymappicine				Ref, 27				
17	camptacumotine				Ref, 28				
18	camptacumanine				Ref, 28				
19	22-hydroxyacuminatine				Ref ,28				
20	angustoline		Ref, 23		Ref, 28				
21	19-hydroxyangustoline				Ref, 29				
22	19-O-methylangustoline				Ref, 29				
23	naucleficine				Ref, 28				
24	dihydrosoquinamine				Ref, 28				
25	venoterpine				Ref, 20	Ref, 21			
26	Vincoside-lactam				Ref, 30				
27	vincosamide	0.092%	0.005%		0.063%	0.05%	0.1%	N	N
28	strictosidinic acid				0.002%			N	N
29	strictosamide	0.002%	0.014%			0.011%	0.035%	N	N
30	pumiloside	0.003%	0.022%			0.02%	0.084%	N	N
31	1-caffeoylquinic acid				Ref, 30				
32	1,2-dihydro-2-oxoquinoline-4-carboxylic acid				Ref, 30				
33	3, 4-methylenedioxy-3'-*O*-methyl-5'-hydroxyellagic acid						<0.001%	N	N
34	3, 3', 4-*O*-trimethylellagic acid	<0.001%				<0.001%	<0.001%	N	N
35	3, 3', 4-*O*-trimethyl-4'-*O*-*β*-D-glucopyranosylellagic acid		<0.001%				<0.001%	N	N
36	3, 4-methylenedioxy-3'-*O*-methyl-4'-*O*-*β*-D-glucopyranosylellagic acid		0.002%				<0.001%	N	N
37	3, 4-methylenedioxy-3', 4'-*O*-dimethyl-5'-methoxyellagic acid	<0.001%					<0.001%	N	N
38	3, 4-methylenedioxy-3', 4'-*O*-dimethyl-5, 5'-dimethoxylellagic acid	<0.001%						N	N
39	3, 4-methylenedioxy-3'-*O*-methyl-5'-hydroxylellagic acid				Ref, 21				
40	3, 4-methylenedioxy-3', 4'-*O*-dimethyl-5, 5'-dimethoxylellagic acid	Ref, 13							
41	3, 4'-O-dimethoxylellagic acid				Ref, 21				
42	3'-*O*-methyl-3,4-*O,O*-methylideneellagic acid				Ref, 21				
43	3, 4-*O*-*O*-methylideneellagic acid				Ref, 21				
44	3',4'-*O*-dimethyl-3,4-*O,O*-methylideneellagic acid				Ref, 21				
45	3,3',4, 4'-tetramethyl-5'-methoxyellagic acid				Ref, 21				
46	5'-hydroxy-3',4'-*O*-dimethyl-3,4-*O,O*-methylideneellagic acid				Ref, 21				
47	3'-*O*-methyl-3,4-*O,O*-metheneellagic acid-4'*O*-*β*-D-glucopynanoside				Ref, 30				
48	quercetin	<0.001%						I: 216.6±21.6	N

49	quercetin-3-*O*-*β*-D-galactopyranoside (hyperoside)	0.028%						I: 132.8±63.8	N
50	quercetin-3-O-*β*-D-glucopyranoside (isoquercitrin)	0.003%						I: 258.7±18.3	N
51	kaempferol-3-O-*β*-D-galactopyranoside (trifolin)	0.03%	0.01%					I: 185.1±26.9	N
52	and kaempferol-3-O-*β*-D-glucopyranoside (astragalin)	0.006%						I: 312.0±12.6	N
53	kaemferol	Ref, 31							
54	camptothin A	Ref, 32							
55	camptothin B	Ref, 32							
56	cornusin A	Ref, 32							
57	gemin D	Ref, 32							
58	tellimagrandin I	Ref, 32							
59	tellimagrandin I	Ref, 32							
60	1,2,6-tri-*O*-galloyl-β-D-glucose	Ref, 32							
61	1,2,3,6-tetra-*O*-galloyl-β-D-glucose	Ref, 32							
62	pedunculagin				Ref, 32				
63	β-sitosterol					Ref, 33			
64	β-sitosterol-3-β-D-glucoside					Ref, 34			
65	methyl ursolate				Ref, 35				
66	methyl betulinate				Ref, 35				
67	betulic acid				Ref, 20				
68	bassic acid	0.01%						N	N
69	amyrin							N	N
70	2,3-dihydroxy-12-ursen-28-oic acid	0.007%						N	N
71	loganic acid				Ref, 30				
72	chlorogenic acid				Ref, 30				
73	gallic acid	Ref, 31							
74	(+)-abscisic acid				Ref, 22				
75	syringic acid				Ref, 21				
76	salicylic acid				Ref, 24				
77	nonandioic acid				Ref, 24				
78	ethyl caffeate				Ref, 35				
79	strycholactone				Ref, 24				
80	sweroside				Ref, 30				
81	inositol				Ref, 30, 35				
82	4-methyl-1,2-cyclonexanedimethanol				Ref, 30				
83	acetamide				Ref, 30				
84	fructose		0.46%						

Notes: Concentrations: numbers in percentage were from the authors and the others were from references (13, 17-35). * Trees were treated by decapitation pruning. Ref with number refers to the reports by other authors. Bioactivity: mean ± S.D.; TOP1 activity: P: Poison; I: Inhibition; N: no activity against TOP1 at 312 μM; blank: no test. Cytotoxicity: Inhibition of PC-3 cell growth at 25 μM; N: <20% inhibition of PC-3 cells at 25 μM. Blank: no test or data available.

To date, 13 CPT derivatives have been identified from various tissues of *Camptotheca* (Table 1). Like CPT, 10-hydroxycamptothecin (HCPT) occurs in all tissues with about 2-10% of the CPT content. All other CPT derivatives are restricted in specific tissues at approximately 0.5-2% of the CPT content. It has been reported that most natural or synthetic CPTs showed potent poison activity against TOP1 (17). Considering their concentrations in plants, however, CPT and HCPT are the major compounds in *Camptotheca* primarily responsible for possible endogenous TOP1 poison activity. Unlike CPTs, however, other active compounds (flavonoids) are unable to poison TOP1 but inhibit free TOP1 enzyme.

Accumulated evidence indicates that the observed abnormal morphogenesis in *Camptotheca* as endogenous autotoxicity is due to induced CPT. The changes of leaf abnormal morphogenesis frequency are in parallel with CPT levels with respect to time. We speculate that if abnormal phenotypes are caused by elevated CPT levels, then it should be possible to suppress the phenotypes by reducing levels of CPT. Indeed, our experimental data support this hypothesis. The abnormal traits appeared with increased CPT and discontinued with the gradual decrease in elevated levels of CPT during the late stage of the experiment (Figure 2*A*, *B*). The CPT induction peak depends on plant age and disturbance intensity. For mature trees, the induced peak of CPT content in intact young tissues appeared in approximately three weeks following light apical pruning (14), and in three or four months following severe decapitation (Figure 2*B*). Abnormal morphogenesis resumed with elevated CPT levels by continuous decapitation pruning. CPT autotoxicity and its induced abnormal morphogenesis disappeared as CPT content decreased. The CPT content is positively correlated with non-entire leaves (r=0.9332) and abnormal morphogenesis (r=0.7095). The direct evidence is that the externally-applied CPT killed apical buds and stem of seedlings and thus results in the induced abnormal morphogenesis in *Camptotheca* seedlings (Figure 3) and CPT-nonproducing red bean seedlings from seeds soaked in higher concentrations of CPT produced similar abnormal morphogenesis (Figure 4).

TOP1 is the only known target of CPTs in eukaryotic organisms, including higher plants (7, 38-41), thus it is believed that CPT's toxic effects on plant growth through its unique effect on TOP1. TOP1 and auxin are

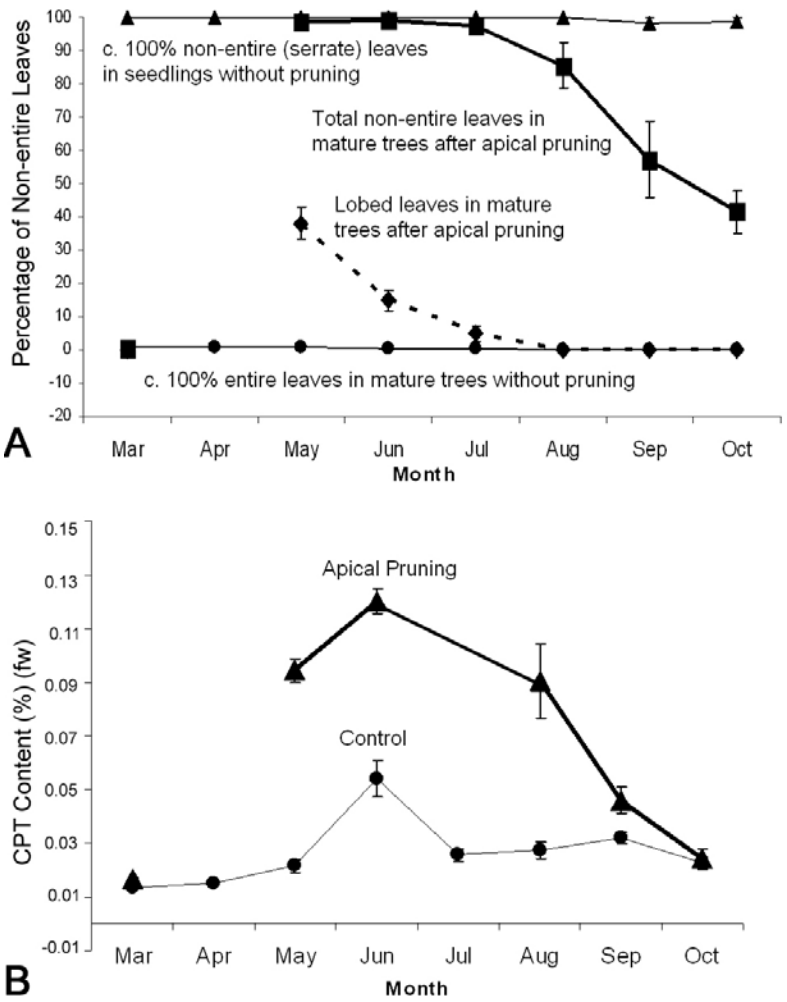

Figure 2. Decapitation induced changes in the leaf margin patterns and CPT content in young leaves (≤2 weeks) of the 6-year-old *Camptotheca acuminata* trees. (A) Leaf margin patterns of plants without disturbance (*triangle*: serrate leaves dominate in the one-year-old wild-type seedlings; *dot*: entire leaves dominate in the 6-year-old wild-type trees); Leaf margin pattern change of the mature trees after pruning treatment in March (*square*: changes of the induced non-entire (serrate and lobed) leaves over time; *diamond*: changes of the induced bidentate, bifid, tridentate, and trifid leaves over time). (B) Changes of CPT contents in young leaves of the 6-year-old mature trees with pruning in March in comparison with untreated trees..

highly concentrated in apical meristems, which control the phenotypes. TOP1 enzyme is highly active in cells that are metabolically active, especially in rapidly dividing tissues such as apical meristems. Auxin is produced in stem apices while CPT is highly accumulated in young fast-growing tissues in all *Camptotheca* varieties (14). Thus, we speculate that the observed serration, lobe of leaves and twisting phyllotaxis in *Camptotheca* following pruning are caused by a disturbance of cell proliferation of meristems. This disturbance might involve the ternary binding complex TOP1-CPT-DNA and the uneven distribution of CPT in space and time within the meristem. The toxicity of CPT *in planta* is also probably carried out through inducing TOP1 gene mutations. At least two genes that encode for TOP1 enzymes, *TOP1*α and *TOP1*β, are found in plants (10). It is reported that the mutation in *TOP1*α caused phyllotaxis and serration of leaf margins in *Arabidopsis* (10), although such morphological abnormalities could also be due to mutation of other genes as such Fas1 and Fas2 (37). The observed resumption of normal morphogenesis following decapitation might be attributed to CPT content decrease to a normal level and reversible TOP1-CPT-DNA complex or DNA repairs following toxicity.

5.2. Auxin reduction as a triggering factor for induced *in vivo* biosynthesis of CPTs

Auxin is an essential plant hormone that regulates diverse aspects of plant growth and development, particularly in controlling apical dominance of a plant and dictating normal phyllotaxis and organogenesis (42). Because apical auxin appears not to enter lateral buds, its inhibition on lateral growth may be via other hormones, for

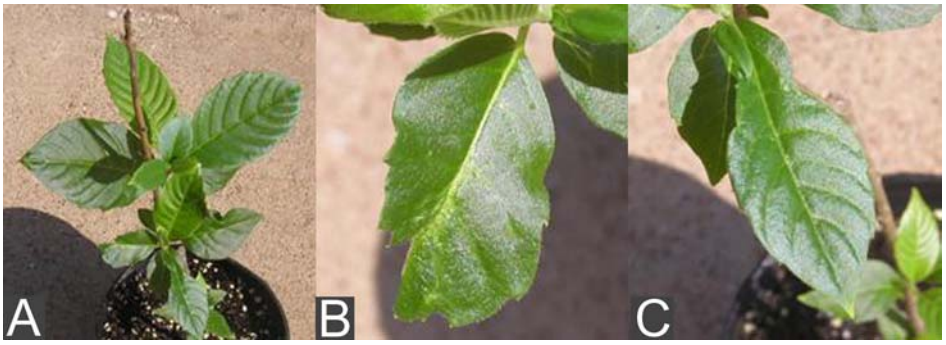

Figure 3. Abnormal morphogenesis of six-month old seedlings of *C. acuminata* following exogenous application of CPT (0.02%, on the basis of dry weight) to apical bud. (A) The whole plant after the CPT application with the back arrow shows the apical bud and stem were poisoned by CPT. (B) Lobed leaf and (C) bifid leaf.

example, by maintaining the level of the bioactive gibberellin (GA_1) by promoting GA_1 biosynthesis and by inhibiting GA_1 deactivation (43). It is commonly known that decapitation has become a common method to stimulate lateral growth in applications and to study auxin's regulation and mechanism (43, 44). Because GA_1 increases from the apex to the stem base in contrast to auxin highly concentrated in apical bud (45), the apical pruning in this study primarily reduced auxin levels in plants although no measurement was made on the auxin reduction caused by decapitation. Endogenous autotoxicity induced by auxin was demonstrated in both auxin reducing and increasing experiments.

5.2.1. Pruning reducing endogenous auxin levels increased CPT contents in plants

In our experiments, decapitation pruning that reduces auxin levels increased CPT several fold (Figure 2*B*) and induced abnormal growth in phyllotaxis and organogenesis in wild-type mature *C. acuminata* (Figure 2*B*), but pruning of lateral stems without significant auxin reduction did not cause autotoxicity in any *Camptotheca* plants. Responding to reduced auxin levels, derivatization of CPT was also enhanced so that we were able to identify several new compounds in the extensively investigated *C. acuminata* following decapitation (46). *Camptotheca* showed two types of changes simultaneously in response to pruning. First, there was a higher frequency of abnormal morphogenesis in the initial new leaf and stem growth after pruning (Figure 2*B*). This endogenous autotoxicity is primarily caused by additional free CPT or its bioactive derivatives. TOP1 enzyme is the primary target of CPTs. However, it is possible that the observed variations in phyllotaxis and leaf margin abnormal morphogenesis of *Camptotheca* might involve more genes as reported in *Arabidopsis* (47). Second, like many other plants, *Camptotheca* has fast lateral growth due to reduced auxin levels following pruning. It is reported that auxin influences the rates of cell division and cell elongation, however, the mechanism remains elusive (37).

5.2.2. Exogenous application of auxin restored auxin-deficient dwarf mutations

Auxin's triggering effect for inducing CPT and abnormal morphogenesis is supported by the following observations in *Camptotheca*. The CPT content of young leaves in different *Camptotheca* varieties exhibits an interesting negative correlation with plant height (Figure 5). *Camptotheca acuminata* that grow to 45 m in height have the lowest CPT content (0.2433% on the basis of dry weight), while the dwarf cultivar *C. lowreyana* 'CT168', which grows to less than 1 m in height when mature, has the highest CPT content (0.5890%). In fact, the auxin-deficient dwarf cultivars of *C. lowreyana* (either 'Katie' or 'CT168') did not have any significant change in CPT content in respond to decapitation. 'CT168' is characterized by a higher than normal CPT content, small heterogeneous leaves, reduced internodes, profuse branching, and major reduction in apical dominance (Figure 1*J*, *L*). A dwarf mutant could be caused by a deficiency in auxin (44, 48). The auxotrophic mutants deficient in hormone biosynthesis can be restored to the wild type by exogenous hormone application (49). Exogenously-fed IAA (indole-3-acetic acid, a primary and naturally occurring auxin in plants) could restore auxin-deficient dwarf mutants (48).

Auxin's triggering effect for inducing CPT is also demonstrated in the restoration of dwarf 'CT168' to a normal phenotype. IBA, originally classified as a synthetic auxin, has been widely used in plant propagation to induce lateral and adventitious roots on cuttings. Since 1985, the presence of IBA as an endogenous auxin has been demonstrated in numerous plants such as *Arabidopsis,* tobacco, maize, carrot, and pea (50, 51). Biochemical evidence in many plants and genetic studies of *Arabidopsis* IBA-response mutants indicate that IBA acts primarily via its conversion to active IAA, which occurs in a mechanism similar to peroxisomal fatty acid β-oxidation (52). It is likely that IBA acts as an IAA precursor, but it is more stable than IAA and acts as a "slow-release" form of IAA (50). Our exogenous application of IBA basally to the cuttings of *Camptotheca* mutant 'CT168' of higher CPT content reduced CPT levels in young leaves by about 60% and stimulated stem elongation and homogeneous leaves so that the plantlets exhibited similar phenotypes as the parent variety. This restoration strongly supports the hypothesis that auxin plays an important role in dwarfism. Further, it suggests that auxin deficiency is a triggering factor for phyllotaxis and leaf margin abnormal morphogenesis. This result is entirely consistent with previous reports that the

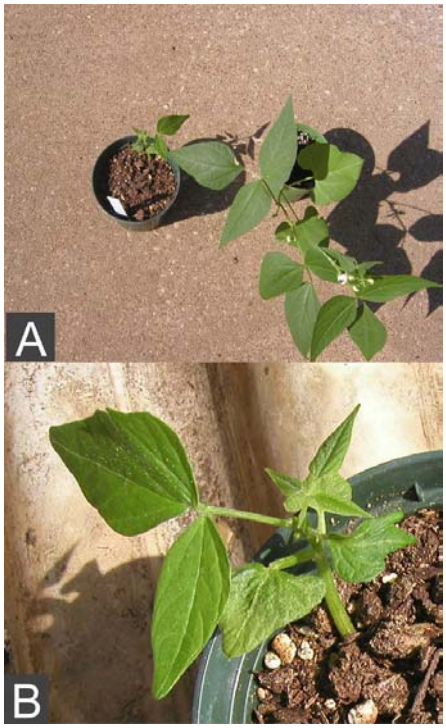

Figure 4. Approximately 16.7% of the red beans (*Phaseolus vulgaris*) germinated from seeds soaked by exogenous CPT at the concentration of 0.02% had one to four abnormal leaves. (A) comparison of normal growing plant from the seeds soaked by water with 10% DMSO for 48 h (left) with the poisoned plant from the seed soaked with 0.02% CPT for 48 h (right). (B) Enlargement of the poisoned plant. In addition to slow growth, the poisoned plants have bifid or lobed leaves vs. normal trifid and entire leaves in untreated plants.

auxotrophic mutants deficient in hormone biosynthesis can be restored to the wild type by exogenous hormone application (44).

5.2.3. Exogenous application of auxin decreased CPT levels in normal plants

In addition to the positive response of CPT to reducing auxin, CPT levels in normal forms of *Camptotheca* plants can be significantly reduced (10-15%) by hydroponically fed IAA (10-100 μg/mL) (14). The inhibition of 1-naphthaleneacetic acid (NAA) on CPT biosynthesis was recently confirmed in a cell culture study of *C. acuminata* (53).

5.2.4. Autotoxicity induced by auxin deficiency rather than auxin homeostasis

Our experiments with *Camptotheca* further suggest that the induced production of CPTs in plants may be caused by auxin reduction rather than auxin homeostasis because (1) the auxin-deficient cultivars have higher CPT contents than normal varieties, (2) exogenous application of auxin reduces CPT levels in plants, and (3) both abnormal morphogenesis and chemical levels returned to normal after auxin homeostasis.

5.3. Defense may play limited roles in induced biosynthesis of CPTs

Current hypotheses to explain how secondary metabolites change after damage can be either supply-side or demand-side, but both emphasize defense function of secondary metabolites, particularly of phytoalexins (54, 55). With high CPT content in young leaves of *Camptotheca* (up to 0.12% on the basis of fresh weight), trees can still grow to 3 m in height annually with normal morphogenesis under natural conditions (14). The role of CPT within *Camptotheca* plants remains elusive. It is commonly believed that CPT content in young leaves is higher because they need more protection (56-59). The young leaves are nutrient-rich and tender in physical structure, thus more attractive to herbivory and pathogenic attacks (56, 57). However, the following evidence does not support the defensive role of CPT in *Camptotheca.*

Endogenous CPT does not protect plants from fungal or herbivory attacks. To date, at least 27 genera of endophytic fungi commonly in CPT-nonproducing plants in China and the United States have been isolated and identified from various tissues of *Camptotheca* (11, 59-62). With CPT at levels of more than 10 times higher than the concentrations to completely inhibit fungal pathogens *in vitro*, young leaves of *Camptotheca* are still seriously infected by at least 10 genera of fungal pathogens, particularly leaf spots (*Alternaria, Epicoccum, Pestalotia*, and *Drechslera*) and root rots (*Fusarium*) (11). The CPT sensitivity of the leaf endophytic fungi *in vitro* assays (11, 59) indicated that CPT is completely or primarily unavailable to the fungi infected *Camptotheca* leaves. In addition to CPT, *Camptotheca* leaves contain much more toxic HCPT at about 10% of the concentration of CPT (Table 1). In fact, *Camptotheca* leaves contain abundant flavonoids that could inhibit the *in vitro* mycelial growth of leaf endophytic fungi as effectively as CPT (11). Similarly, some leaf pests common to the CPT-nonproducing hardwoods in the southern China and United States attack *Camptotheca* leaves, for examples, *Aetora postornata, Cnidocampa flavescens*, and *Parasa* spp. of Limacodidae, *Actias selene* of Saturniidae, *Phassus sinifer* of Hepialidae, and *Hyphantria cunea* of Actiidae (60). A larva of the fall webworm (*Hyphantria cunea*) can consume or damage 10-15 *C. acuminata* leaves a day (60: see figures 37 and 38). The presence of common and CPT-sensitive fungal endophytes and pests in *Camptotheca* indicated that cytotoxic CPT has limited bioactivity *in planta* in *Camptotheca*. In contrast, no fungal infections and pests were observed following decapitation. Probably, the increase of available CPT not only induced endogenous autotoxicity but also protected the plants from attacks.

Camptotheca plants cannot avoid endogenous autotoxicity induced by the increase of their own CPT. This suggests that the plants must fail to balance the toxic CPTs and detoxication *in vivo*. With the accelerated synthesis of toxins, particularly in response to rapid wounding, however, plants may misconduct generation, translocation, perception and transduction of signals and thus, poison themselves. It is reported that the mutation in *TOP1α* caused phyllotaxis and serration of leaf margins in

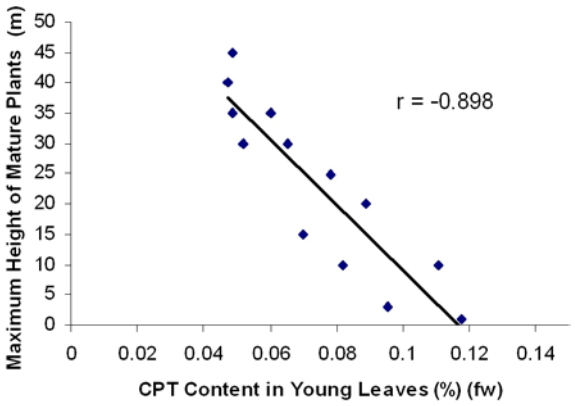

Figure 5. Variation of CPT content of young leaves in different *Camptotheca* taxa.

Arabidopsis (10). TOP1 enzyme is the primary target of CPTs. Recently, it was found that some mutations of *TOP1*s in *Camptotheca* are the mechanism of self-resistance to endogenous CPT (9). The observed auxin-induced variations in phyllotaxis and leaf margin abnormal morphogenesis of *Camptotheca* might be the result of *TOP1* mutations which are caused by induced toxic CPTs. Thus, the induced endogenous toxic CPTs likely have no defensive role in *Camptotheca* and are not desirable for the plants as well. The defensive role of alkaloids CPTs in *Camptotheca* is also challenged by our observations that herbivory, damages, or applications without significantly reducing auxin level did not increase the CPT level in *Camptotheca* (11).

Artemisinin and taxol, two toxic terpenes from plants, may provide additional examples. Artemisinin from herbaceous *Artemisia annua* L. is one of the most widely used antimalarial drugs in the world, and diterpene paclitaxel (taxol) isolated from conifers *Taxus* spp. is one of the commonly used anti-cancer drugs. Both plants and their chemicals are amongst the most extensively investigated, but there is no information on whether it protects the plant against herbivores and pathogens (63). Thus, the role of chemical defense shall not be overemphasized in induced biosynthesis of some toxic secondary metabolites.

5.4. Detoxification and autotoxicity mechanisms in *Camptotheca*

The observed autotoxicity caused by increased endogenous CPT following decapitation and the plant cytotoxicity caused by exogenous CPT suggest that *Camptotheca* plants are sensitive to cytotoxic CPT. Without disturbance, however, plants containing higher CPT content still exhibit fast growth and are not resistant to fungal infections and herbivory. Several common fungal pathogens often seriously infected *C. acuminata* leaves containing high CPT contents and they are clearly not poisoned by CPT in *Camptotheca*. But the isolated fungi can be totally inhibited in culture by CPT at the concentration less than 10% of that naturally occurring in the *Camptotheca* leaves (14). The similar scenario is for larval herbivory. These results imply that toxic CPT *in planta* is primarily not available to the plant cells, infected fungi, insects, and animals that are sensitive to CPT. It has been reported that CPT is accumulated in the glandular trichomes of leaves and stems of *C. acuminata* (58, 63). Subcellular localization studies indicated that CPT accumulates in the vacuole (64). It is likely that *Camptotheca* use the extracellular cavity of glandular trichomes and vacuole for the sequestration of CPT to keep the majority of the toxic compounds away from the target (DNA TOP1) of plant cells and thus plant self-toxicity is avoided.

Another possibility is that some CPT may occur in plants as inactive derivatives, but the reactivity of these inactive compounds during isolation may make the detection of original forms difficult. The attachment of a sugar to the free toxic alkaloid inactivates the chemical and thus, acts as a detoxication mechanism of plants (1, 2). These bounding forms are reversible to alkaloids during hydrolysis. The balance between active free CPTs and the inactive bounding forms in living plants (limited bioavailability of free CPTs *in planta*) along with the balance between active TOP1 and free CPTs in living plants provide two rational explanations for the avoidance of self-poison or detoxication in *Camptotheca*. However, no CPT glycosides have been isolated from *Camptotheca* leaves. Detoxification mechanisms of CPT-producing plants, particularly the role and frequency of TOP1 mutations and existence of CPT in non-toxic forms needs further investigation. It is also important to identify TOP1 genes from the leaves and stems with abnormal morphogenesis and determine their roles in endogenous autotoxicity.

Without revelation of detoxification mechanisms in *Camptotheca*, it is hard to elucidate how plants get self-poisoned by endogenous toxic CPT. Understanding the relationship between auxin and CPT may help us to explain the observed endogenous autotoxicity in *Camptotheca*. As discussed above, CPT contents in *Camptotheca* can be increased by reducing auxin levels but decreased by the exogenous addition of auxin. Further, CPT might have inhibitory effects on IAA as reported for hypaphorine and other indole alkaloids (65). The mechanism of interaction between auxin and CPT has not been elucidated. IAA is synthesized both from tryptophan (Trp) using Trp-dependent pathways and from an indolic Trp precursor via Trp-independent pathways during normal growth (51). Plants may switch from basal Trp-independent IAA biosynthesis to Trp-depedent pathways during stress, when more IAA may be needed (66, 67). *Camptotheca* may use the Trp-dependent route to synthesize IAA following plant wounding. Trp-dependent biosynthesis is also a major source for CPT (68). IAA and CPT share the same Trp-dependent pathways during stress and this could be a starting point for the investigation of their relationship (Figure 6).

During normal plant development, CPT may be synthesized in young leaves, roots and bark because strictosamide and pumiloside, two key biosynthetic intermediates of CPT are primarily found in these tissues

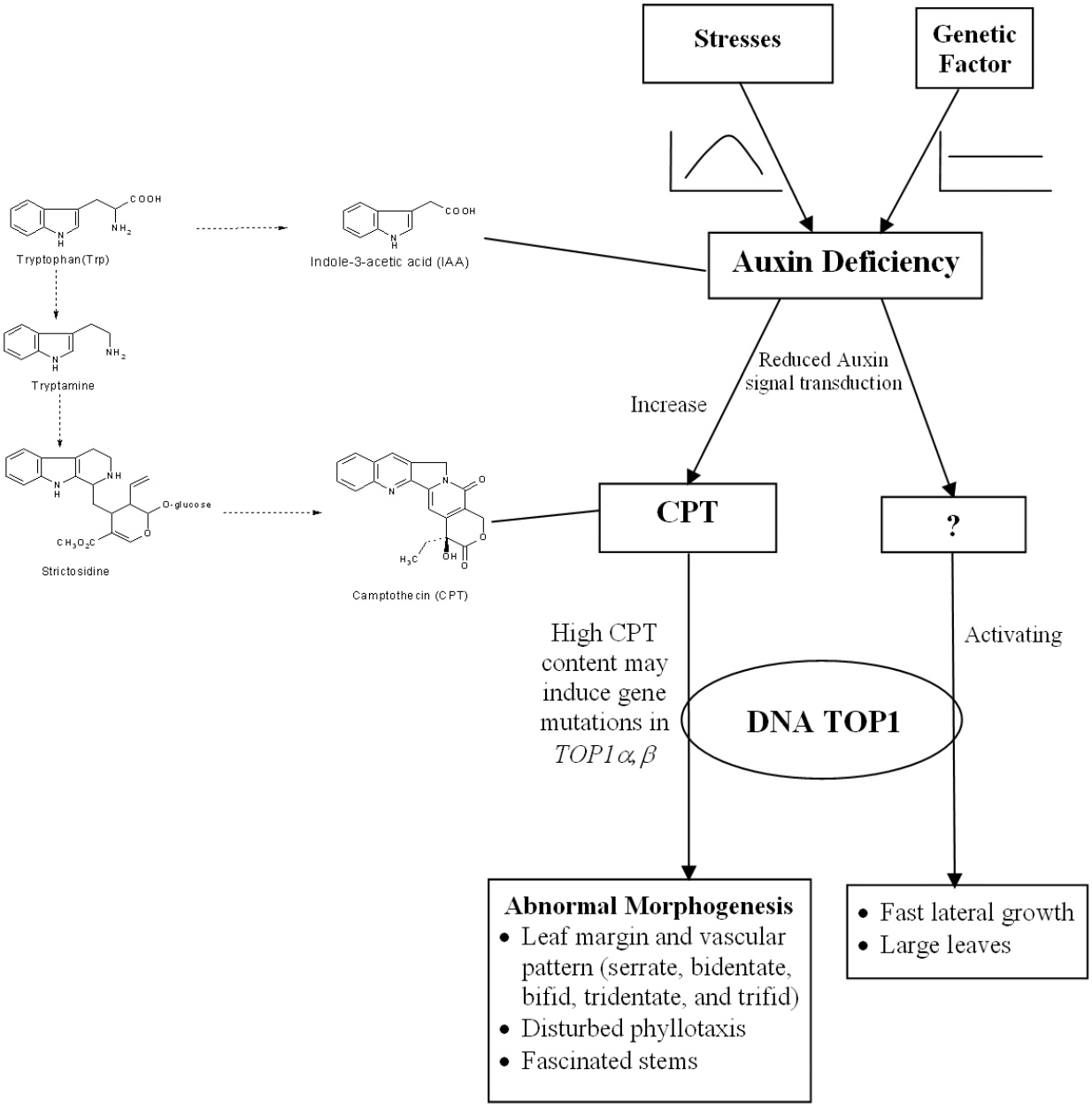

Figure 6. Proposed scheme of endogenous autotoxicity induced by auxin deficiency following decapitation pruning in *Camptotheca*. Auxin deficiency can impact *Camptotheca* phenotype by two ways at the same time (1) to induce abnormal morphogenesis by producing more free CPT to poison TOP1 enzyme or genes (endogenous autotoxicity); and (2) to stimulate fast plant growth by reducing inhibition through unknown mechanism. The two contrary routes may occur in other plants producing TOP1 inhibiting metabolites. Auxin deficiency caused by environmental stresses is temporary and induced endogenous autotoxicity disappears as soon as the elevated CPT levels decrease with discontinuation of disturbance. Cultivars deficient in auxin keep higher CPT contents consistently and thus inherit abnormal phenotype by asexual reproduction. Tryptophan, a common biosynthetic precursor during stress may be a key to understand relation between IAA and CPT.

but not in wood or fruits (Table 1). Then CPT may accumulate in leaf trichomes and cell vacuoles or is segregated from these tissues to other accumulation tissues such as fruits. Following apical decapitation, however, strictosamide and pumiloside are primarily in root bark (approximately 150% and 280% higher than the concentrations in stem bark, respectively). Also, seven CPT derivatives including three compounds are found exclusively in root bark after decapitation (Table 1). This implies that CPTs may be produced primarily in roots following decapitation. CPT is transported to new apical meristems from roots, through trunk and stems, in its own

form or possibly in the form of soluble molecules. The new leaves and stems develop abnormal morphogenesis due to *TOP1* poisoned by newly synthesized active CPT or derivatives during their transportation from roots. No abnormal morphogenesis occurs following light pruning. This may be due to the maintenance of a normally high auxin signal, which induces CPT to be produced mainly in young leaves, despite the fact that some terminal tissues were removed. In addition to induced autotoxicity, plants may have some detoxification mechanisms following decapitation. A non-toxic CPT glycoside, 20-β-D-glucopyranosyl 18-hydroxycamptothecin was recently isolated from the roots of *C. acuminata* after decapitation but not in other tissues (46) (Table 1).

It is possible that plants producing other TOP1-inhibiting metabolites may have similar routes while others may have different routes. This explains why decapitation pruning has not been reported to cause plant morphological abnormality, even though it is commonly known than pruning stimulates growth. Unlike environmental stresses (e.g., drought), decapitation pruning can increase biomass growth, CPT content, and derivatization (14). Thus, auxin-reducing pruning is an effective method to enhance CPT yield in *Camptotheca*.

6. ACKNOWLEDGEMENTS

We thank Y.Y. Yi, Y.J. Wang, Z.Z. Zhang, and W.L. Zhang for their assistance in data collection, and D. Creech, D. Kulhavy, F.C. Shi, and S. Ownby for comments on the manuscript. This work was partially supported by Camptotech, Inc., Houston Livestock Show and Rodeo, Fondren Foundation, J. Hicks, Mr. and Mrs. D. Dolben, C. Poland, U.S. Department of Education P116Z040020, U.S. Centers for Disease Control and Prevention R01 CI000315-01, and U.S. Department of Agriculture 2008-03460 to S.Y.L.

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Abbreviations: CCK-8: Cell Counting Kit-8; CPT: camptothecin; HCPT: 10-hydroxycamptothecin; GA_1**:** gibberellin; IBA: Indole-3-butyric acid; IAA: indole-3-

acetic acid; PC-3: Human prostate cancer; NAA: 1-naphthaleneacetic acid; TOP1: DNA topoisomerase I; *TOP1α* and *TOP1β*: two genes that encode for TOP1 enzymes; Trp: tryptophan; WST-8: water soluble tetrazolium

Key Words: Endogenous Autotoxicity, Decapitation Pruning, Auxin Deficiency, Camptothecin, DNA Topoisomerase I, *Camptotheca*

Send correspondence to: Shiyou Li, Box 6109, SFA Station, Nacogdoches, TX 75962, USA, Tel: 936-468-2071, Fax, 936-468-2489, E-mail: lis@sfasu.edu

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